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Jasmonic Acid Signaling Modulates Ozone-Induced Hypersensitive Cell Death

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Recent studies suggest that cross-talk between salicylic acid (SA)–, jasmonic acid (JA)–, and ethylene-dependent signaling pathways regulates plant responses to both abiotic and biotic stress factors. Earlier studies demonstrated that ozone (O_3) exposure activates a hypersensitive response (HR)–like cell death pathway in the Arabidopsis ecotype Cvi-0. We now have confirmed the role of SA and JA signaling in influencing O_3-induced cell death. Expression of salicylate hydroxylase (NahG) in Cvi-0 reduced O_3-induced cell death. Methyl jasmonate (Me-JA) pretreatment of Cvi-0 decreased O_3-induced H_2O_2 content and SA concentrations and completely abolished O_3-induced cell death. Cvi-0 synthesized as much JA as did Col-0 in response to O_3 exposure but exhibited much less sensitivity to exogenous Me-JA. Analyses of the responses to O_3 of the JA-signaling mutants *jar1* and *fad3/7/8* also demonstrated an antagonistic relationship between JA- and SA-signaling pathways in controlling the magnitude of O_3-induced HR-like cell death.

INTRODUCTION

Plants continuously produce active oxygen species (AOS) such as superoxide radical ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$) as a consequence of normal cellular metabolism. Under normal conditions, plants rapidly metabolize these AOS with the help of constitutive antioxidant enzymes or metabolites (Scandalios, 1997). However, when subjected to environmental stresses such as cold, high light, ozone (O_3), pathogens, and UV irradiation, excess AOS is generated. This increase in AOS production necessitates the activation of additional defenses (Doke, 1997; Scandalios, 1997). Unless these AOS are efficiently metabolized, they rapidly oxidize and damage membrane lipids, proteins, and other cellular components. This leads to cellular dysfunction and can ultimately cause cell death, which is manifested by the appearance of necrotic lesions (Mudd, 1997). Based on the assumption that AOS are highly reactive and lead to cellular dysfunction, AOS generation is frequently considered deleterious and harmful. However, recent studies have demonstrated that AOS are important components of signaling pathways that influence plant defense responses, including cell death (Jabs et al., 1996; Alvarez et al., 1998; Karpinski et al., 1999; Solomon et al., 1999).

H_2O_2 and $^{\bullet}O_2^-$ have been implicated as signal molecules that can activate defense responses. Treating cell cultures with H_2O_2 alone induces defense responses and cell death (Levine et al., 1994; Solomon et al., 1999). However, although sublethal H_2O_2 concentrations induce expression of defense genes, complete induction of defense genes and cell death requires additional signaling molecules such as salicylic acid (SA) at the whole-plant level (Chamnongpol et al., 1998; Rao and Davis, 1999). These studies have established that AOS probably require additional downstream components to transduce or amplify the signal (Van Camp et al., 1998; Bolwell, 1999). Among several molecules proposed to act downstream of AOS, SA, jasmonic acid (JA), and ethylene are considered major regulators of plant defense responses (Dong, 1998; Glazebrook, 1999). SA is one of the most widely studied stress-signaling molecules; its role in influencing plant resistance to pathogens and other stress factors is well documented (Draper, 1997; Shirasu et al., 1997; Surplus et al., 1998; Rao and Davis, 1999). Similar to SA, JA also is believed to play an important role in influencing plant resistance to pathogens and other stress factors (Creelman and Mullet, 1997; Penninckx et al., 1998; Vijayan et al., 1998). The recent cloning of *COI1* has increased our understanding of the involvement of protein degradation in JA-induced responses (Xie et al., 1998); however, little is

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known about other potential modes of action of JA-dependent signaling pathways in controlling plant defense.

A cyclopentanone, JA influences plant growth and development as well as plant responses to different stress factors such as pathogens and wounding (Creelman and Mullet, 1997; Staswick et al., 1998; Vijayan et al., 1998). One of the major roles postulated for JA is its antagonistic action on SA-dependent signaling pathways (Creelman and Mullet, 1997; Seo et al., 1997). Some published evidence also indicates that, depending on the plant species and stimulus, SA and JA can act either antagonistically or synergistically in controlling stress responses. For example, JA antagonizes SA-dependent stress-induced accumulation of acidic pathogenesis-related (PR) proteins (Niki et al., 1998), whereas SA antagonizes stress-induced JA biosynthesis and JA-mediated signaling (Pena-Cortes et al., 1993; Doares et al., 1995). In other experiments, however, simultaneous application of methyl jasmonate (Me-JA) and SA resulted in synergistic activation of *PR1* gene expression in tobacco (Xu et al., 1994), and treating plants with 2,6-dichloro isonicotinic acid, a functional analog of SA, induced JA-dependent defense gene expression in rice plants (Schweizer et al., 1997). Likewise, ethylene potentiates SA-mediated induction of *PR1* in Arabidopsis (Lawton et al., 1994), whereas *NPR1* is required to activate the ethylene- and JA-mediated systemic resistance induced by *Pseudomonas fluorescens* (Pieterse et al., 1998).

By virtue of its chemical properties, O_3 spontaneously dissociates in leaf extracellular spaces, generating such AOS as $^{\bullet}O_2^-$ or H_2O_2 (Mudd, 1997; Runeckles and Vaartnou, 1997). Having shown that O_3 mimics the oxidative burst generated during the early stages of the hypersensitivity response (HR) and that it can act as an elicitor of plant defense responses (Rao and Davis, 1999), we have used O_3 as an agent to study the downstream components of AOS-responsive signaling pathways. O_3 reacts primarily with the plasma membrane, causing alterations in lipid composition and increasing the production of linoleic acid (Mudd, 1997), a precursor of JA biosynthesis (Creelman and Mullet, 1997). Thus, it is possible that O_3 triggers JA biosynthesis and that JA plays a role in influencing plant responses to O_3 or other oxidative stress–generating stimuli (Kangasjarvi et al., 1994). The possible involvement of JA in O_3-induced responses is supported by the observation that exogenous application of JA or wounding before exposure to O_3 reduces O_3-induced cell death in tobacco (Orvar et al., 1997) and hybrid poplar (Koch et al., 2000) and decreases the O_3-induced concentrations of H_2O_2 in tobacco (Schraudner et al., 1998). However, the mode of action by which JA influences O_3-induced cell death is not known. Having shown that SA concentrations play an important role in modulating the type and the magnitude of O_3-induced cellular defenses and cell death (Sharma et al., 1996; Rao and Davis, 1999), we investigated the role of JA in influencing O_3-induced responses. Here, we present results demonstrating that JA signaling plays an important role in modulating an O_3-induced, SA-dependent pathway for HR cell death.

RESULTS

Compromising SA Accumulation Reduces the O_3 Sensitivity of Cvi-0

Considering the documented role of SA in activating the HR cell death pathway (Draper, 1997; Shirasu et al., 1997), we proposed that an O_3-induced oxidative burst could trigger SA biosynthesis and potentiate a cell death pathway that initiates lesion formation through the same mechanism that regulates HR. Our earlier studies—demonstrating that the O_3-sensitive ecotype, Cvi-0, accumulates high concentrations of SA and that treatment of Cvi-0 plants with diphenylene iodinium, a suicide inhibitor of membrane-localized oxidases, diminished the magnitude of O_3-induced cell death (Rao and Davis, 1999)—support this hypothesis. To test whether increased concentrations of SA were required for O_3-induced cell death in Cvi-0, we generated transgenic Cvi-0 plants expressing a bacterial *nahG* gene encoding the SA-degrading enzyme salicylate hydroxylase (Delaney et al., 1994). A previously described conductivity assay was used to obtain a more quantitative measure of the decrease in cell death exhibited by Cvi-0:NahG plants (Rao and Davis, 1999). As shown in Figure 1, exposure of Cvi-0 plants to 300 ppb O_3 for 4 hr caused a conductivity increase of 2.4-fold. This O_3-induced increase in conductivity was ~40% less in Cvi-0:NahG plants than in Cvi-0 plants. These results clearly show that compromising SA content reduced the magnitude

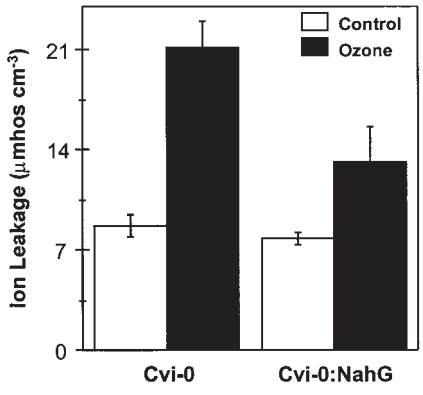

Figure 1. *nahG* Expression in Cvi-0 Reduces O_3-Induced Cell Death.

Plants were exposed to a single dose of 300 ppb O_3 for 4 hr as described in Methods. Plants maintained in O_3-free air served as controls. Leaf discs were excised, briefly rinsed in water, and incubated in 5 mL of distilled water for 6 hr, after which the conductivity of the solution was measured with a conductivity meter. Three independent transgenic lines were tested, and the data from one representative line are presented. The experiment was performed twice with two replicates in each. Error bars represent ±SE ($n = 4$).

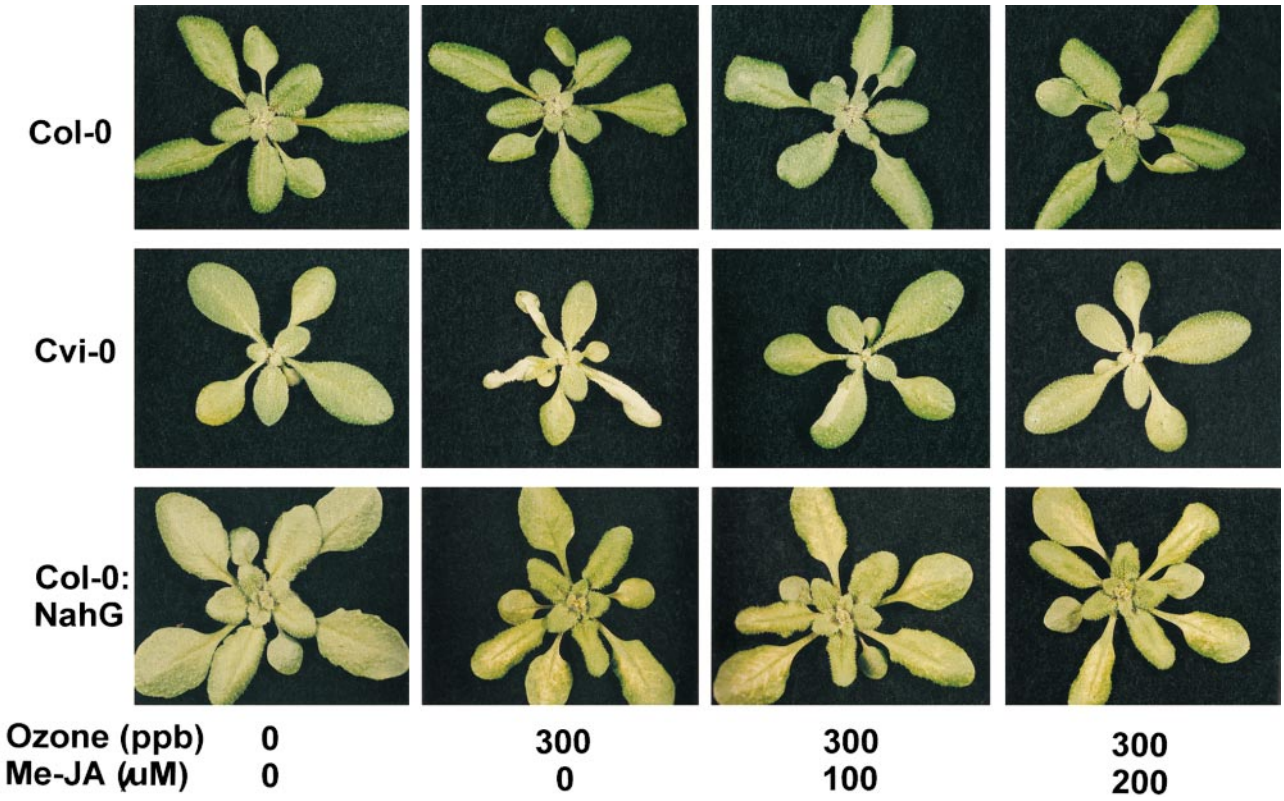

Figure 2. Me-JA Pretreatment Abolishes O_3-Induced Cell Death.

Plants treated with various concentrations of Me-JA 22 hr before exposure to O_3 were exposed to a single 300-ppb dose of O_3 for 6 hr. Plants were allowed to recover in O_3-free air for an additional 16 hr after the O_3 exposure before being scored for visible injury symptoms. Plants sprayed with 0.1% alcohol alone and maintained in O_3-free air served as controls. Plants exposed to O_3 alone were also sprayed with 0.1% alcohol to normalize the spraying effect. The experiments were performed twice, and the plants presented are from a representative experiment.

of O_3-induced lesions in Cvi-0 and are consistent with the hypothesis that the O_3 sensitivity of Cvi-0 is attributable to activation of an SA-dependent cell death pathway.

Me-JA Pretreatment Decreases O_3-Induced Cell Death

Our earlier studies demonstrated that O_3 exposure activates two distinct cell death pathways in Arabidopsis. Compromising the accumulation of SA in Col-0:NahG plants reduced cellular defenses and caused a cell death response in O_3-treated plants that was distinct from the O_3-induced oxidative burst, hyperaccumulation of SA, and activation of the HR cell death pathway observed in Cvi-0 (Rao and Davis, 1999). Because wounding the plant or applying JA before the exposure to O_3 diminished O_3-induced cell death in tobacco (Orvar et al., 1997) and hybrid poplar (Koch et al., 2000), we tested whether Me-JA (a biologically active derivative of JA) also would affect O_3-induced cell death in Arabidopsis.

As shown in Figure 2, Col-0 exposed to O_3 alone did not develop foliar lesions; however, both Col-0:NahG and Cvi-0 plants developed lesions with distinct kinetics and severity. Cvi-0 developed large lesions within 6 to 8 hr after the initiation of O_3 exposure, whereas Col-0:NahG plants developed smaller lesions within 24 hr after initiation (Rao and Davis, 1999). Pretreating plants with various concentrations of Me-JA 22 hr before exposure to O_3 attenuated O_3-induced cell death in Cvi-0 in a dose-dependent manner (Figure 2). A concentration of 200 μM Me-JA was sufficient to completely abolish O_3-induced necrotic lesions in Cvi-0 (Figure 2). In contrast to Cvi-0, Me-JA treatment of Col-0:NahG plants affected neither the kinetics nor the magnitude of O_3-induced cell death (Figure 2). These findings suggest that Me-JA specifically affects O_3-induced HR cell death in Cvi-0 but does not affect the cell death exhibited by Col-0:NahG plants.

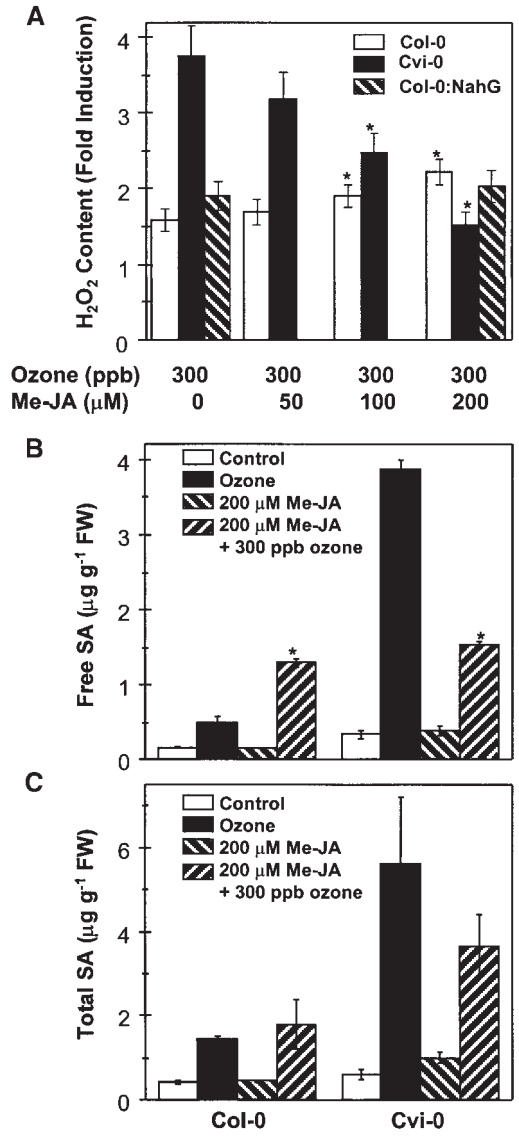

Figure 3. Me-JA–Mediated Modulation of O_3-Induced H_2O_2 Content Is Correlated with Changes in SA Accumulation.

Plants treated with various concentrations of Me-JA 22 hr before exposure to O_3 were exposed to a single 300-ppb dose of O_3 for 6 hr. Afterward, samples were frozen in liquid nitrogen and used to determine the concentrations of H_2O_2 and SA, as described in Methods. H_2O_2 and SA contents of plants treated with <200 μM Me-JA were identical to those of plants treated with 200 μM Me-JA (data not shown). Plants sprayed with 0.1% alcohol alone and maintained in O_3-free air served as controls. Plants exposed to O_3 alone were also sprayed with 0.1% alcohol to normalize the spraying effect.

(A) H_2O_2 content.

(B) Free SA content.

(C) Total SA content.

The values presented are the mean values of two different experiments with two replicates in each. Asterisks indicate significant differences compared with the appropriate control plants exposed to O_3 alone ($P < 0.05$). FW, fresh weight of tissue. Error bars indicate ±SE ($n = 4$).

Me-JA Pretreatment Differentially Modulates O_3-Induced H_2O_2, SA Contents, and Defense Gene Expression in Col-0, Cvi-0, and Col-0:NahG

Our studies with Cvi-0 suggested that an SA-mediated feedback amplification loop resulting from excess production of AOS and SA and expression of defense genes (Draper, 1997; Shirasu et al., 1997) activated an HR-like cell death pathway with O_3-induced lesion formation (Rao and Davis, 1999). Because Me-JA pretreatment attenuated O_3-induced cell death only in the Cvi-0 plants, we tested whether Me-JA treatment would affect the O_3-induced oxidative burst, SA accumulation, and subsequent defense gene expression. Compared with untreated control plants, foliar concentrations of H_2O_2 in O_3-tolerant Col-0 plants exposed to O_3 for 6 hr increased by 59%, whereas the H_2O_2 concentrations in O_3-sensitive Col-0:NahG and Cvi-0 plants increased by 90% and 3.7-fold, respectively (Figure 3A). Treatment of plants with Me-JA alone did not influence foliar H_2O_2 content in these genotypes. However, Me-JA pretreatment increased the magnitude of O_3-induced H_2O_2 concentrations in Col-0 but attenuated the O_3-induced H_2O_2 in Cvi-0 in a dose-dependent manner compared with plants exposed to O_3 alone (Figure 3A). No major changes were observed in foliar H_2O_2 concentrations of Col-0:NahG plants irrespective of the treatment.

Similar to its effects on foliar H_2O_2 contents, pretreatment of Col-0 with 200 μM Me-JA more than doubled the concentrations of free SA; the same pretreatment of Cvi-0 decreased the O_3-induced accumulation of free SA by 65% compared with plants exposed to O_3 alone (Figure 3B). Similar significant changes were also observed for total SA accumulation (Figure 3C). These results suggest that Me-JA pretreatment specifically attenuates an O_3-induced oxidative burst in Cvi-0 but has no effect on the modest increase in H_2O_2 that occurs during necrosis in Col-0:NahG.

Earlier studies demonstrated that both AOS and SA, depending on their amounts present, influence cellular defense gene expression and HR cell death (Chamnongpol et al., 1998; Rao and Davis, 1999). Me-JA treatment attenuated O_3-induced H_2O_2 and SA concentrations in Cvi-0 but increased the magnitude of O_3-induced H_2O_2 and SA content in Col-0. Hence, we tested whether Me-JA pretreatment affected O_3-induced defense gene expression by monitoring the steady state transcript levels of both SA-dependent and SA-independent defense genes. Consistent with our earlier reports, O_3 exposure for 6 hr caused a significant induction of SA-dependent *GST1* transcripts (Figure 4A) and *PR1* transcripts (Figure 4B) in Cvi-0 (by 21.4- and 23-fold, respectively) and in Col-0 (by 16.4- and 11.6-fold, respectively), whereas these transcripts were induced very little or not at all in Col-0:NahG plants (4.5- and 0-fold; Figure 4A). Although pretreating the plants with Me-JA did not influence the magnitude of O_3-induced *GST1* transcript levels (Figure 4A) or protein concentrations (Figure 4D) in any of the genotypes tested, pretreatment of Col-0 and Cvi-0 attenuated the magnitude of O_3-induced *PR1* transcripts in a dose-

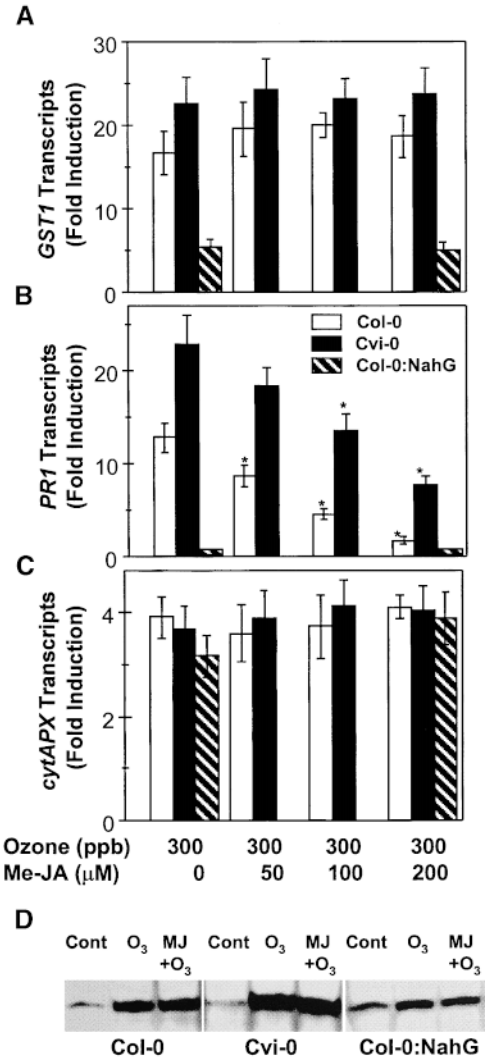

Figure 4. Me-JA Pretreatment Specifically Attenuates O_3-Induced, SA-Dependent Expression of the *PR1* Gene.

Plants in **(A)** to **(C)** were treated with various concentrations of Me-JA 22 hr before exposure to O_3 and then exposed to a single 300-ppb dose of O_3 for 6 hr. After the completion of 6 hr of O_3 exposure, samples were frozen in liquid nitrogen and used for RNA gel blot analysis as described in Methods. Total RNA was isolated, fractionated on a 1% formaldehyde-agarose gel, transferred to a nylon membrane, and hybridized with a ^{32}P-labeled gene-specific probe. The amount of hybridized radioactivity was measured by using a PhosphorImager, and the data presented were corrected for loading differences in RNA by reprobing the filters with a control rRNA probe (Rao and Davis, 1999). Plants sprayed with 0.1% alcohol alone and maintained in O_3-free air served as controls. Plants exposed to O_3 alone were also sprayed with 0.1% alcohol to normalize the spraying effect. The experiments were repeated twice, and the *n*-fold change in the mean values obtained relative to their respective controls are shown. The relative counts ($\times 10^{-4}$) for *GST1*, *PR1*, and *cytAPX* were 1.06 ± 0.42, 1.2 ± 0.38, and 20.2 ± 3.21, respectively, in control Col-0 plants; 1.206 ± 0.49, 1.8 ± 0.52, and 29.9 ± 4.4, respectively, in Cvi-0 plants; and 1.00 ± 0.65, 0.67 ± 0.12, and 20.57 ± 5.12, respectively, in Col-0:NahG plants. The relative counts of plants treated with 200 μM Me-JA alone were not significantly different from the plants treated with 0.1% alcohol (data not shown). Col-0:NahG plants were not tested for *GST1* mRNA accumulation at 50 and 100 μM Me-JA.

(A) Relative changes in SA-dependent *GST1* mRNA transcripts compared with those in control plants.

(B) Relative changes in SA-dependent *PR1* mRNA transcripts compared with those in control plants.

(C) Relative changes in SA-independent *cytAPX* mRNA transcripts compared with those in control plants.

(D) Gel blot analysis of GST1 protein. Plants were treated with 200 μM Me-JA 22 hr before exposure to O_3 and then exposed to a single 300-ppb dose of O_3 for 6 hr. After 6 hr of exposure, the plants were allowed to recover in O_3-free air for 4 hr (10 hr after the initiation of O_3 exposure), and samples frozen in liquid nitrogen were used for protein gel blot analysis as described in Methods. Equal amounts of protein (50 μg) were electrophoresed and blotted onto the membrane, in which they were probed with anti-GST antiserum as described in the text. The experiment was repeated twice, and the digitized image from one representative experiment is shown. Cont, plants treated with 0.1% alcohol; O_3, plants treated with 0.1% alcohol and exposed to 300 ppb O_3 for 6 hr; MJ+O_3, plants treated with 200 μM Me-JA and exposed to 300 ppb O_3 for 6 hr. No major differences were observed in the GST1 protein content of plants treated with 200 μM Me-JA alone and those treated with 0.1% alcohol; hence, only the GST1 protein content from control plants is shown.

Asterisks in **(A)** to **(C)** indicate significant differences in comparison with the appropriate control plants exposed to O_3 alone ($P < 0.01$); error bars indicate ±SE ($n = 3$).

dependent manner (Figure 4B). The O_3-induced *PR1* transcript levels of Col-0 and Cvi-0 pretreated with 200 μM Me-JA were between 15 and 35% of the values detected in plants exposed to O_3 alone. Irrespective of the treatment, no major changes were observed in the very low *PR1* transcript level in Col-0:NahG plants (Figure 4B).

To test whether Me-JA treatment influenced other characterized O_3-induced, SA-independent events, we examined O_3-induced *cytAPX* mRNA accumulation. A 6-hr O_3 exposure induced increases in the transcript level of a *cytAPX* in Col-0, Cvi-0, and Col-0:NahG plants by 3.6-, 3.3-, and 3.2-fold, respectively, over that of control plants. As shown in Figure 4C, treating plants with various concentrations of Me-JA up to 200 μM did not influence the magnitude of O_3-induced *cytAPX* mRNA in any of the three genotypes. These findings suggest that Me-JA pretreatment attenuated O_3-induced cell death without affecting the magnitude of some O_3-induced, SA-dependent, and SA-independent antioxidant defense gene expression.

Cvi-0 Has Reduced Sensitivity to Me-JA

Exogenous application of Me-JA attenuated O_3-induced H_2O_2 and SA content and completely reduced O_3-induced

cell death in a dose-dependent manner in Cvi-0 (Figures 2 and 3). Hence, we hypothesized that Cvi-0 could have a defect in either stress-induced JA biosynthesis or JA perception (or both), a defect resulting in increased activation of a SA-dependent HR-like cell death pathway that is ultimately responsible for the increased O_3 sensitivity exhibited by this ecotype. We explored this possibility by measuring the amounts of transcript of allene oxide synthase (*AS*), a key enzyme in JA biosynthesis, the JA content, and the mRNA levels of a molecular marker for responsiveness to JA, the vegetative storage protein AtVSP (Creelman and Mullet, 1997; Staswick et al., 1998). To monitor the changes in *AS* transcripts, we used reverse transcription–polymerase chain reaction (RT-PCR) with gene-specific primers based on published sequence information (Laudert et al., 1996). O_3 exposure caused a rapid increase in the expected 1.6-kb *AS* RT-PCR product in both Col-0 and Cvi-0 (Figure 5A), whereas the constitutive control *Act2* (1.2 kb) was unaffected by O_3 treatment (Figure 5B). Consistent with the increase in *AS* transcripts, O_3 exposure enhanced JA content in both Col-0 and Cvi-0 with similar kinetics and magnitude. A marked increase in JA content was observed in both Col-0 and Cvi-0 within the first 3 hr of O_3 exposure, and a 6-hr O_3 exposure enhanced the JA content of those strains by 13.6- and 12.5-fold, respectively, over that of control plants (Figure 5C). These findings suggest that Cvi-0 is capable of synthesizing as much JA as Col-0 is, if not more, and thus is not deficient in O_3-induced JA biosynthesis.

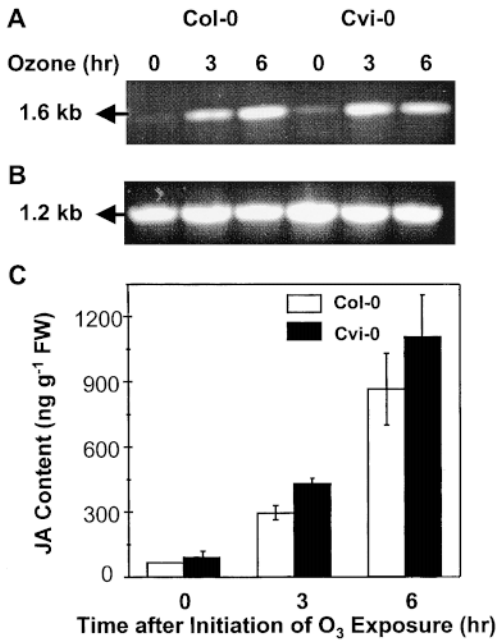

Figure 5. O_3 Exposure Increases JA Accumulation.

Plants were exposed to a single dose of 300 ppb O_3 for 6 hr; at the indicated intervals, samples were collected and used for RNA isolation or JA measurements as described in Methods. RT-PCR was performed with gene-specific primers as described, and a 10-μL portion of the reaction mixture was separated on 0.8% agarose gel. The image was digitized and edited with Adobe Photoshop (5.0). The experiments shown in **(A)** were repeated with total RNA isolated from an independent experiment with *Act2* as a control in **(B)**. The image from one representative experiment is shown. Size lengths of the PCR fragments are shown at left.

(A) RT-PCR analysis of *AS*.

(B) RT-PCR analysis of actin (*Act2*).

(C) JA content. Values are the mean values of three different experiments. FW, fresh weight. Error bars indicate ±SE ($n = 3$).

We next explored the possibility that Cvi-0 might be insensitive to JA, monitoring the expression patterns of *AtVSP* in Me-JA–treated plants. *AtVSP* transcripts were easily detected in the untreated control plants of Col-0, but no visible hybridized signal was observed in Cvi-0. The results shown in Figure 6 for analyses using different Me-JA concentrations reveal that Me-JA treatment induced *AtVSP* transcripts in Cvi-0 in a dose-dependent manner but less than in Col-0 (Figure 6A). Treating Col-0 plants with 50 μM Me-JA increased *AtVSP* transcripts by 16-fold, whereas the induction seen in Cvi-0 was marginal and always lower than the amount of *AtVSP* transcripts detected in untreated Col-0 (Figure 6B). Analysis of the time dependence of this response by using high concentrations of Me-JA (200 μM) further revealed that *AtVSP* transcript induction in Cvi-0 occurred at a much lower level with slower kinetics than observed in Col-0 (Figure 6C). These results demonstrate that, compared with Col-0, Cvi-0 has much less sensitivity to JA.

JA-Signaling Mutants Have Greater Sensitivity to O_3

The results of these experiments are consistent with the hypothesis that the reduced sensitivity of Cvi-0 to Me-JA may have resulted in a concomitant hyperaccumulation of SA to amounts sufficient to trigger cell death, resulting in apparent O_3 sensitivity. However, this interpretation is based on comparisons with Col-0, and these results instead may be due to differences in the genetic background of the two different ecotypes rather than directly related to JA signaling. To further test whether JA or JA signaling modulates the magnitude of O_3-induced HR cell death, we took advantage of the availability of JA mutants in a Col-0 background. Earlier studies have shown that Col-0 exposed to O_3 for extended periods (3 to 4 days) did not exhibit lesion formation (Sharma and Davis, 1994). Figure 7 shows that exposure of *jar1*, a JA-insensitive mutant, and *fad3/7/8*, a mutant blocked in JA biosynthesis, to 300 ppb O_3 for 6 hr resulted in the rapid development of large lesions. *jar1* exposed to O_3 developed lesions within 8 hr, *fad3/7/8* within 12 to 14 hr after the initiation of O_3 exposure. These findings further indicate that JA-dependent signaling pathways play an important role in influencing O_3-induced cell death.

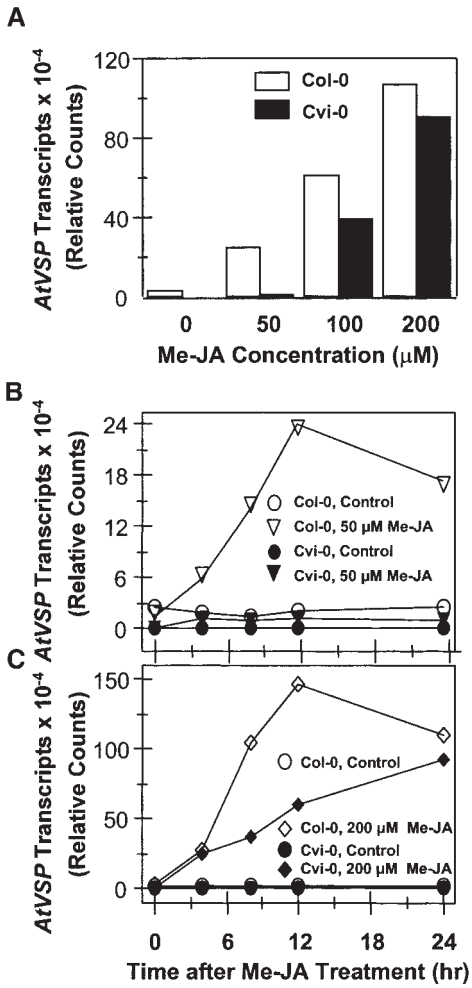

Figure 6. Cvi-0 Has Low Sensitivity to Me-JA Treatment.

Plants were treated with 0.1% alcohol (control) or with 50, 100, or 200 μM Me-JA for the indicated periods. RNA isolation and gel blot analyses were performed as described for Figure 4. Note that the *y* axes in **(B)** and **(C)** have different scales. The experiments were repeated twice with similar results; the data presented are from a representative experiment.

(A) Dose-dependent response of *AtVSP* transcripts to Me-JA treatment for 24 hr.

(B) Kinetics of *AtVSP* transcript induction by 50 μM Me-JA treatment.

(C) Kinetics of *AtVSP* transcript induction by 200 μM Me-JA treatment.

O_3 Sensitivity of *jar1* Is Correlated with Increased O_3-Induced AOS, SA Accumulation, and Defense Gene Expression

Because *jar1* rapidly developed lesions in response to O_3 exposure, we tested whether the O_3-induced cell death in *jar1* is associated with greater H_2O_2 and SA contents, as had been observed previously in Cvi-0. Figure 8A shows that a 6-hr O_3 exposure of Col-0 and *jar1* increased foliar H_2O_2 concentrations by 1.6- and 3.0-fold, respectively, compared with those of control plants. H_2O_2 levels were similar to those of control plants in Col-0 24 hr after the initiation of O_3 exposure, whereas the H_2O_2 levels of *jar1*, although reduced, were still substantially higher compared to Col-0. Similar to the results observed with Cvi-0, *jar1*:Col-0 exposed to O_3 accumulated 11.8- and 16.2-fold greater concentrations of total and free SA, respectively, compared with the corresponding 3.2- and 2.5-fold increases observed in Col-0 (Figure 8B). Analysis of *PR1* transcript contents revealed that a 6-hr O_3 exposure increased *PR1* transcripts in Col-0 and *jar1* by 8.8- and 29.5-fold, respectively, compared with control plants (Figure 8C). These results strongly suggest that JA insensitivity increased the magnitude of O_3-induced, SA-mediated responses in Arabidopsis in an ecotype-independent manner.

DISCUSSION

Cross-Talk between Multiple Signaling Pathways Influences Plant Defense Responses

The outcome of the interaction of plants with a given stress is governed by several factors, including the genotype, the physiological state of the plant, the presence of other stress

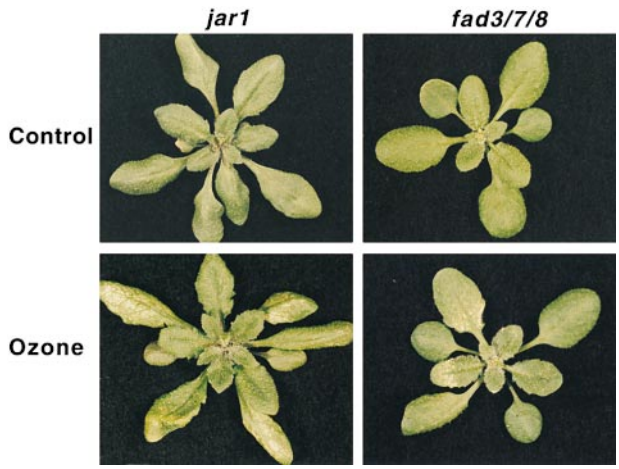

Figure 7. JA Mutants Are Highly Sensitive to O_3.

JA mutants (*jar1* and *fad3/7/8*) were exposed to a single dose of 300 ppb O_3 for 6 hr. Plants were allowed to recover in ambient air conditions for 24 hr after the initiation of O_3 exposure before they were scored for visible injury symptoms. Plants maintained in O_3-free air served as controls. The experiment was conducted twice, and the plants shown represent typical leaf damage.

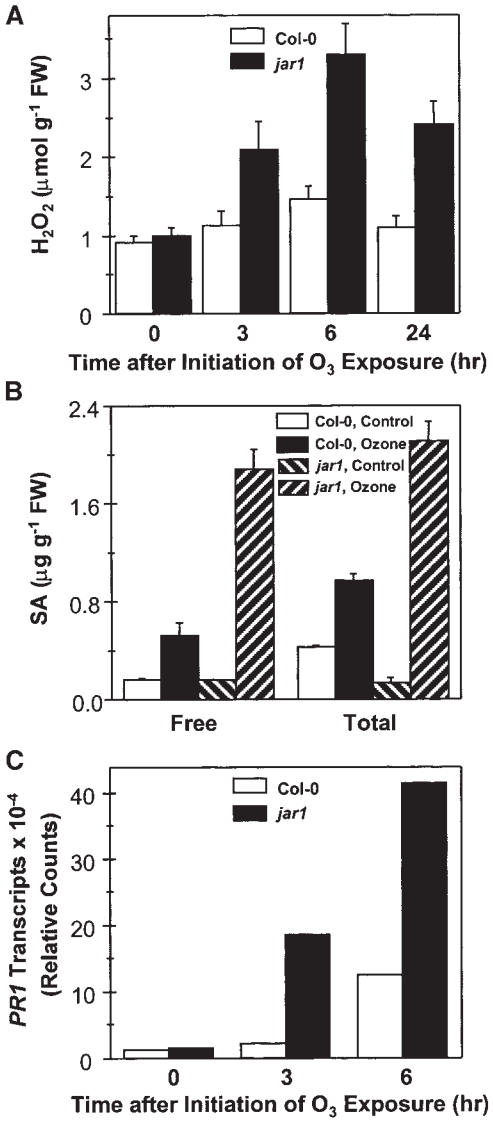

Figure 8. Insensitivity to JA Increases the Magnitude of O_3-Induced Oxidative Stress and SA Biosynthesis.

Plants were exposed to a single dose of 300 ± 50 ppb O_3 for 6 hr and allowed to recover for as long as 24 hr in ambient air after the initiation of the O_3 exposure. **(A)** and **(B)** show the mean values of three different experiments; error bars indicate ±SE ($n = 3$).

(A) Kinetics of H_2O_2 accumulation.

(B) SA content.

(C) *PR1* gene expression. The relative counts from one representative experiment of repeated experiments are shown. FW, fresh weight of tissue.

factors and environmental signals, and any specific interactions that might occur between the activated signaling pathways. Among various signaling molecules proposed to modulate stress responses, AOS, SA, JA, and ethylene are widely believed to be the global regulators of plant defense responses (Dong, 1998; Bolwell, 1999; Jabs, 1999). Although SA, JA, and ethylene activate distinct signaling pathways, recent studies suggest that the SA- and JA-dependent pathways interact and that this extensive cross-talk influences the magnitude or amplitude of the other pathway (Seo et al., 1997; Shah et al., 1999). For example, overexpression of a rice Ras-like G protein gene, *rgp1,* or a tobacco mitogen-activated protein kinase gene rendered SA accumulation and *PR1* gene expression responsive to wounding (Sano et al., 1994; Seo et al., 1995). In addition, *NPR1/NIM1*, a gene controlling SA-mediated systemic acquired resistance (SAR) (Dong, 1998), has been shown to be essential for both JA- and ethylene-mediated activation of SAR induced by *P. fluorescens* (Pieterse et al., 1998). More recently, Shah et al. (1999) showed that *SSI1* might function as a molecular switch to modulate the expression of SA- and JA-dependent pathways. These studies clearly established that SA-, JA-, and ethylene-dependent pathways do not act independently and that key regulatory components may control the cross-talk between these signaling pathways.

JA-Signaling Pathways Attenuate SA-Dependent HR-like Cell Death

In the present study, we obtained direct evidence for the role of JA signaling in regulating the magnitude of O_3-induced, SA-dependent HR cell death that bears strong similarities to the HR activated during plant–pathogen interactions. Our studies with the highly O_3-sensitive Arabidopsis ecotype, Cvi-0, demonstrated a relationship between JA insensitivity (Figure 6), increased accumulation of H_2O_2 and SA (Figure 3), and activation of a cell death pathway in response to O_3 exposure (Figure 2). Exogenous application of Me-JA attenuated O_3-induced content of H_2O_2 and free SA by >50% and abolished cell death in Cvi-0 in a dose-dependent manner. These results support the hypothesis that JA signaling pathways modulate the magnitude of SA-mediated HR in O_3-exposed plants. The specificity of Me-JA in influencing SA-dependent HR-like cell death was demonstrated by the inability of Me-JA to affect O_3-induced H_2O_2 concentrations (Figure 3A) and necrosis in Col-0:NahG plants (Figure 2). Our previous studies have established that the antioxidant defense responses of Col-0:NahG plants exposed to O_3 were greatly diminished, resulting in the accumulation of toxic metabolites that likely contribute to cell death by way of a mechanism distinct from HR cell death (Rao and Davis, 1999). Thus, the ability of jasmonates to provide tolerance to O_3 exposure appears to be limited to cases in which lesion formation is the result of activation of an SA-dependent cell death pathway that is likely to be the same pathway activated during the HR.

Additional direct evidence indicating that JA signaling pathways modulate the magnitude of O_3-induced, SA-mediated cell death came from the analysis of JA mutants in a Col-0 background. Col-0 is relatively resistant to O_3 (Sharma

and Davis, 1994), but the *jar1* and *fad3/7/8* mutants rapidly developed lesions in response to O_3 (Figure 7). Detailed analyses further revealed that *jar1* accumulated high amounts of H_2O_2 and SA, concomitant with increased expression of the *PR1* gene (Figure 8) before the formation of lesions (Figure 7). Analysis of another JA-insensitive mutant, *coi1* (Xie et al., 1998), also revealed a similar increase in the magnitude of SA-mediated O_3-induced responses as compared with wild-type Col-0 (data not shown).

The timing of Me-JA application was an important factor in influencing O_3-induced cell death. Pretreating Cvi-0 with Me-JA for 3 or 6 hr before treatment had no major effect on O_3-induced cell death, whereas treating the plants with Me-JA for 22 hr before O_3 exposure had the most effect (data not shown). This is consistent with analyses of Me-JA–induced *AtVSP* transcripts in Cvi-0 (Figures 6B and 6C), which demonstrated that high concentrations of Me-JA (200 μM) are required to induce a response in Cvi-0 comparable to that in Col-0 and that Cvi-0 takes >12 hr to reach those levels. In contrast to the results obtained in experiments with the tobacco cv Bel W3, wounding Cvi-0 at 3 or 12 hr before exposure to O_3 did not affect O_3-induced cell death (data not shown). That probably reflects the inability of wounding to promote JA accumulation sufficient to overcome the JA insensitivity exhibited by Cvi-0.

JA Signaling Pathways Act Both Upstream and Downstream of SA Accumulation

Our data clearly suggest that disturbances in the JA signaling pathway increased the magnitude of an SA-dependent HR cell death pathway. How can the increased SA biosynthesis in JA-insensitive plants be explained? O_3 reacts primarily with plasma membranes and, in some cases, causes increased lipoxygenase activities and concentrations of linoleic acid, a major precursor of JA biosynthesis (Kangasjarvi et al., 1994; Mudd, 1997). JA is known to rapidly inhibit the expression of genes involved in photosynthesis, induce chlorophyll loss, and induce cellular changes that ameliorate photochemical damage (Bunker et al., 1995; Creelman and Mullet, 1997). Thus, during oxidative stress such as that imposed by O_3 exposure, this lipoxygenase-mediated generation of JA could attenuate the severity of oxidative stress (Creelman and Mullet, 1997). Support for this hypothesis is provided by the observation that the basal and O_3-induced amounts of the mRNA of the JA-regulated gene *AtVSP* were considerably higher in Col-0 than those observed in Cvi-0 and *jar1*:Col-0 plants (data not shown). Thus, insensitivity to JA, such as that found in Cvi-0 and *jar1*:Col-0 plants, may interfere with the JA-dependent modulation of the O_3-induced oxidative burst, resulting in greater concentrations of SA in these plants than in wild-type Col-0. Because these elevated SA levels exceed the threshold limit required to initiate cell death, lesions form under conditions that do not induce lesions in Col-0.

Analyses of Cvi-0 and *jar1*:Col-0 plants indicated that insensitivity to JA increased the magnitude of O_3-induced H_2O_2 and stimulated the SA-dependent HR pathway; they also suggested that JA affects O_3-induced cell death upstream of SA accumulation. However, analysis of the responses of Col-0 identified a second potential control point acting downstream of SA accumulation but upstream of *PR1* induction. Although Col-0 pretreated with 200 μM Me-JA and exposed to O_3 accumulated H_2O_2 and free SA in quantities that were 40% and twofold greater, respectively, than in plants exposed to O_3 alone (Figures 2 and 3), the amount of *PR1* transcripts was only 10 to 15% of that detected in plants exposed to O_3 alone (Figure 5). Studies with *lsd6* and *lsd7* mutants have indicated that cell death acts downstream of SA accumulation (Weymann et al., 1995). Studies of *phx* mutations, which suppress the *lsd5* mutant phenotype, have demonstrated a direct correlation between the transcript levels of the SA-dependent gene *PR1* and cell death (Morel and Dangl, 1999). The fact that Me-JA treatment increased the magnitude of O_3-induced SA content in Col-0 but decreased SA-dependent *PR1* transcript levels suggests that JA signaling pathways can differentially regulate the magnitude of the defense responses typically associated with the HR. This dual role of JA signaling pathways in regulating O_3-induced H_2O_2 concentrations (upstream of SA) and the SA-mediated HR pathway (downstream of SA) may explain the increased O_3 sensitivity of JA mutants.

In our studies, Me-JA treatment completely abolished cell death in Cvi-0, with little effect on the magnitude of expression of O_3-induced SA-dependent *GST1* and SA-independent *cytAPX* (Figure 4). Similarly, the numbers of O_3-induced *GST1* and *cytAPX* transcripts were almost identical in Col-0, *jar1*:Col-0, and Cvi-0 plants (Rao and Davis, 1999; data not shown). These results suggest that increased JA signaling reduces SA signaling below the threshold required to activate the HR cell death without affecting the magnitude of antioxidant-based induction of defense genes. This is consistent with earlier studies demonstrating that greater amounts of AOS, SA, or both are required to activate cell death than are required for defense gene expression (Levine et al., 1994; Chamnongpol et al., 1998; Rao and Davis, 1999).

Is Cvi-0 Allelic to Other JA-Insensitive Mutants?

The unexpected discovery that Cvi-0 has greatly reduced JA sensitivity raised the possibility that the major O_3-sensitivity locus in Cvi-0 may be allelic to other known JA-insensitive mutants, particularly because these mutants also exhibit increased sensitivity to O_3. Furthermore, our current mapping data placed the O_3-sensitivity locus of Cvi-0 in close proximity to *coi1*:Col-0 and *jar1*:Col-0. Thus, the increased sensitivity to O_3 and the decreased JA responses exhibited in Cvi-0 may indicate that an allele of one of these loci confers insensitivity to JA. If such is the case, however, this Cvi-0 allele has very distinct properties because several phenotypes exhibited by Cvi-0 are quite different from those observed in

coi1:Col-0 and *jar1*:Col-0 mutants. For example, Cvi does not have the JA-resistant root growth phenotype exhibited by *coi1*:Col-0, *jar1*:Col-0, or *jin*:Col-0 mutants (data not shown). In addition, the O_3-sensitive phenotype of Cvi-0 can be rescued with exogenous treatment of Me-JA, whereas Me-JA treatment failed to rescue the O_3-sensitive phenotype of *jar1*:Col-0 (data not shown). In contrast to *coi1*:Col-0, which is male sterile, Cvi-0 is completely fertile. Our ongoing genetic tests for allelism should clarify the relationship of the O_3-sensitivity of Cvi-0 to these JA signaling loci.

Conclusions

Our studies have shown that independent linear signaling cascades do not control O_3-induced plant defense responses; instead, components of one pathway can affect signaling through other pathways. We propose the general model shown in Figure 9, which suggests that JA signaling pathways reduce O_3-induced cell death by attenuating the O_3-induced oxidative burst (upstream of SA accumulation; step 1) and SA-mediated HR cell death pathway (downstream of SA accumulation; step 2). In O_3-tolerant Col-0 plants, O_3 induces both SA and JA pathways, which then act antagonistically. This interaction may allow plants to modulate the relative amount of SA- and JA-inducible defenses as a function of time in response to a specific stress. The increased activation of JA signaling pathways in Col-0 may minimize the magnitude of an O_3-induced oxidative burst, resulting in production of an amount of SA adequate for triggering sufficient defense gene expression to confer resistance (Figure 9, step 1) but not high enough to trigger cell death (Figure 9, step 2). In Cvi-0 and *jar1*:Col-0, the diminished sensitivity to JA may increase the magnitude of oxidative burst, resulting in increased SA biosynthesis and subsequent SA-mediated potentiation of the HR cell death. Thus, JA appears to attenuate the biosynthesis of SA and subsequent SA-mediated cell death. This ability of JA to attenuate cell death processes could be critical in situations in which cell death has no apparent benefit for survival or plant productivity. Therefore, identification of specific mechanisms that regulate the interaction of SA- and JA-dependent signaling pathways with each other, as well as with other signaling pathways, will be indispensable for improving plant resistance to biotic and abiotic stresses.

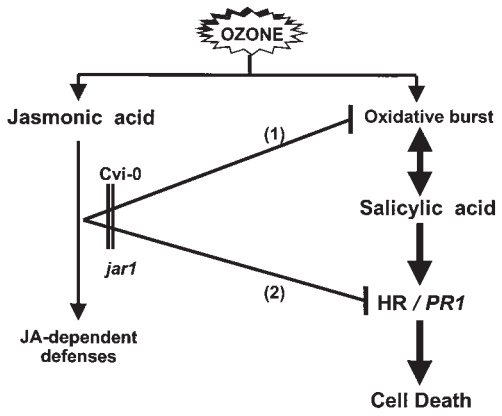

Figure 9. Schematic Model Illustrating the Potential Role of JA Signaling Pathways in Modulating the Magnitude of O_3-Induced, SA-Mediated Responses.

Both SA and JA signaling pathways are activated in response to O_3 exposure. JA signaling can attenuate the magnitude of the O_3-induced oxidative burst (upstream of SA accumulation; step 1) and SA-mediated HR cell death pathway (downstream of SA accumulation; step 2). Therefore, plant resistance to oxidative stress stimuli is dependent on SA and JA signaling pathways that then act antagonistically; this allows plants to modulate the relative amount of SA- and JA-inducible defenses, including cell death, as a function of time in response to a specific stress.

METHODS

Plant Growth and O_3 Treatments

Arabidopsis thaliana accessions Col-0 (Columbia), Cvi-0, and *jar1*:Col-0 were obtained from the Arabidopsis Biological Resource Center (ABRC; Ohio State University, Columbus, OH). The Col-0:NahG transgenic line expressing bacterial salicylate hydroxylase, and the *fad3/7/8* and *coi1* mutants in Col-0 background, were obtained from Drs. John Ryals (formerly at Novartis), John Browse (Washington State University, Pullman, WA), and John Turner (University of East Anglia, UK), respectively. Because *coi1* and *fad3/7/8* are male sterile, rosette leaves were collected separately from individual plants during experimental treatments, and the plants were allowed to grow until seed set. Individuals that did not form siliques were assumed to have the *coi1* and *fad3/7/8* genotype, and only tissues from these plants were subjected to further analysis. Plants were grown in growth chambers at 22/18°C (day/night) temperature, 50 to 70% relative humidity, and a photosynthetic photon flux density (PPFD) of 120 μmol m^{-2} sec^{-1} with a 16-hr photoperiod according to our standard conditions described elsewhere (Rao and Davis, 1999).

Unless indicated otherwise, 18-day-old plants were exposed to a single dose of 300 $\pm$ 50 ppb O_3 for 6 hr (9:00 AM to 3:00 PM) in an O_3 chamber as previously described (Rao and Davis, 1999). The environmental conditions in the O_3 chamber averaged 22/18°C (day/night) temperature, 50 to 60% relative humidity, and a PPFD of 110 μmol m^{-2} sec^{-1} with a 16-hr photoperiod. Desired O_3 concentrations were generated with an O_3 generator (model O3-21; O_3 Associates, Kensington, CA) and were monitored with a UV Photometric O_3 analyzer (model 49C; Thermo Environmental Instruments Inc., Franklin, MA). Plants maintained in O_3-free air served as controls.

All H_2O_2 measurements, RNA gel blot analyses, reverse transcription–polymerase chain reaction (RT-PCR), and protein gel blot analysis were done with the third and fourth rosette leaves randomly collected from six to eight different plants. Salicylic acid (SA) and jasmonic acid (JA) measurements were taken by pooling whole rosettes

from 15 to 20 plants. Samples were flash-frozen in liquid nitrogen and stored at −80°C until further analyses. Significant differences between mean values were evaluated with a commercial statistical analysis package (SigmaStat; Jandel Scientific, Chicago, IL).

Methyl Jasmonate Treatment

A stock solution of methyl jasmonate (Me-JA; 100 mM) was first made up in absolute alcohol; various concentrations of Me-JA (50, 100, and 200 μM) were prepared by appropriate dilution in water and adjusted to the final concentration with 0.1% alcohol. Seventeen-day-old plants were sprayed with Me-JA 22 hr before O_3 exposure with a commercial sprayer until runoff and were maintained in separate chambers until exposure to O_3. Plants sprayed with 0.1% alcohol alone and maintained in O_3-free air served as controls. Plants exposed to O_3 alone were also sprayed with 0.1% alcohol to normalize for any spraying effects.

Visible Injury

To assess the changes in visible injury, we exposed plants treated with various concentrations of Me-JA or 0.1% alcohol (control) to 300 ± 50 ppb O_3 for 6 hr. Plants were allowed to recover in O_3-free air for 24 hr after the initiation of O_3 exposure before they were scored for visible injury. Plants maintained in O_3-free air served as controls.

Transgenic Cvi-0 and O_3-Induced Cell Death

Dr. John Ryals (Delaney et al., 1994) kindly provided a construct that contains the bacterial *nahG* gene encoding salicylate hydroxylase in the pTJS75 binary vector. This NahG construct was transformed into an *Agrobacterium tumefaciens* strain GV3101 and was used to transform Cvi-0 according to the method described by Bechtold et al. (1993), except that vacuum, Murashige and Skoog medium, and benzylamino purine were not included in the transformation protocol. Three independent kanamycin-resistant lines were allowed to self, and the resulting progeny were retested for kanamycin resistance. At least 10 kanamycin-resistant progeny derived from each of the three independent transformants were allowed to self. These seeds too were retested to identify lines that appeared to be homozygous on the basis of 100% of the seedlings being kanamycin resistant. Progeny derived from crosses of these homozygous lines with Col-0 exhibited kanamycin resistance at a 3:1 ratio (resistant:sensitive), indicating that the three lines used in these studies had single T-DNA insertion sites. Twenty-day-old Cvi-0 and Cvi-0:NahG plants were exposed to a single dose of 300 ppb O_3 for 4 hr, and O_3-induced cell death was assayed by measuring ion leakage from leaf discs. A total of eight leaf discs, 1 cm in diameter, were excised from the third and fourth rosette leaves of six different plants, briefly rinsed in water, and floated on 5 mL of double-distilled water for 6 hr at room temperature. The conductivity of the water was measured with a conductivity meter (model 1051; Amber Science, San Diego, CA).

H_2O_2 Measurements

Frozen leaves (0.2 g) were ground to a powder under liquid nitrogen and homogenized with 1 mL of 0.2 M $HClO_4$ in a precooled pestle and mortar. The extract was held on ice for 5 min and centrifuged at 10,000*g* for 10 min at 4°C. The supernatant was collected and either processed immediately or quick-frozen at −80°C until further analysis. All analysis was completed within 72 hr of extraction, a period in which no substantial autooxidation of H_2O_2 was observed. The acidic supernatant was neutralized to pH 7.0 to 8.0 with 0.2 M NH_4OH, pH 9.5, and briefly centrifuged at 3000*g* for 2 min to sediment the insoluble material. The colored components in the extract were removed by applying the extract (0.5 mL) to a 2-mL column of AG 1X-8 resin (Bio-Rad) and eluting with 3 mL of double-distilled water. H_2O_2 concentrations were measured essentially by the method of Warm and Laties (1982). Briefly, 50 μL of the cleared extract was mixed with 845 μL of 0.2 M NH_4OH, pH 9.5, and 5 μL of 5 mM luminol in a test tube and placed in the measurement chamber. The luminescence was detected after automated injection of 100 μL of 0.5 mM potassium ferricyanide over a 5-sec period with a luminometer (model LB 9501; Berthold System Inc., Pittsburgh, PA). H_2O_2 concentrations were obtained by calibrating the counts to a standard graph generated with known amounts of H_2O_2 treated essentially as described above.

SA and JA Measurements

Whole rosettes of 15 to 20 plants were pooled for the estimation of both free and total SA as described by Lee and Raskin (1998). All data were corrected for recovery (85%) by including internal controls. JA was analyzed according to established protocols (Creelman and Mullet, 1995).

RNA Isolation, RNA Gel Blot Analysis, RT-PCR, and Gene Probes

Total RNA was isolated using a SDS–phenol extraction method and subjected to RNA gel blot hybridization analysis as previously described (Rao and Davis, 1999). Because we found the message abundance of allene oxide synthase (AS) to be too low for definitive characterization by normal RNA gel blot analysis, we obtained semiquantitative data for *AS* expression by RT-PCR with a GeneAmp RT-PCR kit (Perkin-Elmer, Norwalk, CT). Total RNA (10 μg) treated with DNase I (Gibco-BRL, Gaithersburg, MD) was reverse-transcribed with an antisense primer (5′-CTAAAAGCTAGCTTTCCTTAACGACGA-3′) in a total volume of 20 μL according to the manufacturer's instructions. Then, 5 μL of the RT reaction product was used as a template in a 50-μL PCR assay with a sense primer (5′-ATGGCTTCTATTTCAACCTTTCCG-3′). These sense and antisense primers were designed to amplify a 1.6-kb product based on a published sequence (Laudert et al., 1996). For the PCR amplification and loading controls, the same template cDNA was amplified using primers for the constitutive actin (*Act2*) gene as described by Li et al. (1998). Twenty cycles with an annealing temperature of 56°C were performed. Ten microliters of the PCR product was separated on an agarose gel; the image obtained was digitized and edited with Adobe Photodeluxe (version 5.0; San Jose, CA). Control studies were performed to optimize for linearity with respect to template quantity, and the amount of PCR product amplified under these conditions was found to be directly proportional to the amount of input RNA (data not shown).

The probe for *cytAPX* was kindly provided by Dr. Hiraku Saji (National Institute of Environmental Studies, Tsukuba, Japan). A cDNA encoding *AtVSP* (ABRC clone 108B11T7) was obtained from the ABRC as a result of a BLAST search (Altschul et al., 1997) using

known sequence information for *AtVSP*. *GST1* and *PR1* probes were as described earlier (Rao and Davis, 1999). The clones obtained from the ABRC were sequenced before use to confirm their identity.

Protein Gel Blot Analysis

For protein gel blot analysis, the third and fourth rosette leaves from three different plants were flash-frozen in liquid nitrogen 10 hr after the initiation of exposure to O_3 and stored at −80°C until analyzed. Soluble proteins were extracted by homogenizing frozen leaf tissue in 100 mM potassium phosphate buffer, pH 7.5, containing 2 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. Protein content was measured according to Bradford (1976) with BSA as a standard. Equal amounts of protein (50 μg) were electrophoresed by 10% SDS-PAGE, and electrophoretically transferred (12 V for 18 hr at 4°C) to Hybond nitrocellulose membrane (Amersham). The filters were blocked with 5% nonfat milk in 1 × Tris-buffered saline (TBS, 20 mM Tris-HCl and 150 mM NaCl, pH 8.0) for 2 hr at room temperature and then incubated for 1 hr at room temperature with a rabbit glutathione-S-transferase-1 antiserum at a final dilution of 1:1000. The antiserum was diluted in 1 × TBS containing 5% nonfat milk, and the filters were rinsed three times with 1 × TBS containing 0.1% Triton X-100 (TBS-T) for 10 min each. Filters were incubated for 45 min with the horseradish peroxidase–conjugated secondary antibody diluted 1:5000 in 1 × TBS containing 1% nonfat milk then rinsed four times with TBS-T for 10 min each; the reaction was developed for 20 sec with a chemiluminescence kit (Amersham) according to the manufacturer's instructions. The image was digitized and edited in Adobe Photoshop (5.0). Protein gel blot analysis was repeated twice, and an image from a representative experiment is presented.

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