Ozone-Sensitive Arabidopsis rcd1 Mutant Reveals Opposite Roles for Ethylene and Jasmonate Signaling Pathways in Regulating Superoxide-Dependent Cell Death

Kirk Overmyer,a Hannele Tuominen,a Reetta Kettunen,a Christian Betz,b Christian Langebartels,b Heinrich Sandermann, Jr.,b and Jaakko Kangasjärvi,a,1

a Institute of Biotechnology and Department of Biosciences, University of Helsinki, POB 56 (Viikinkkaari 5 D), FIN-00014 Helsinki, Finland
b Institute of Biochemical Plant Pathology, GSF Research Center for Environment and Health, D-85764 Oberschleissheim, Germany

We have isolated a codominant Arabidopsis mutant, radical-induced cell death1 (rcd1), in which ozone (O3) and extracellular superoxide (O2•−), but not hydrogen peroxide, induce cellular O2•− accumulation and transient spreading lesions. The cellular O2•− accumulation is ethylene dependent, occurs ahead of the expanding lesions before visible symptoms appear, and is required for lesion propagation. Exogenous ethylene increased O2•−-dependent cell death, whereas impairment of ethylene perception by norbornadiene in rcd1 or ethylene insensitivity in the ethylene-insensitive mutant ein2 and in the rcd1 ein2 double mutant blocked O2•− accumulation and lesion propagation. Exogenous methyl jasmonate inhibited propagation of cell death in rcd1. Accordingly, the O3-exposed jasmonate-insensitive mutant jar1 displayed spreading cell death and a prolonged O2•− accumulation pattern. These results suggest that ethylene acts as a promoting factor during the propagation phase of developing oxyradical-dependent lesions, whereas jasmonates have a role in lesion containment. Interaction and balance between these pathways may serve to fine-tune propagation and containment processes, resulting in alternate lesion size and formation kinetics.

INTRODUCTION

Human activities have increased tropospheric ozone (O3) concentration two- to fivefold during the past 40 years (Kley et al., 1999). O3 poses a twofold challenge to plants: photosynthesis and growth can be impaired by increased background concentrations, whereas acutely phytotoxic O3 concentrations are additionally manifested as foliar lesions in sensitive species and cultivars (Kangasjärvi et al., 1994; Kley et al., 1999). Both of these modes of O3 action potentially result in crop losses worth several million dollars annually. Historically, O3 has been regarded as a “wound stress” that causes necrosis by oxidizing and damaging plasma membranes (reviewed in Heath and Taylor, 1997). Recent results, however, indicate that O3 responses resemble components of the hypersensitive response (HR) found in incompatible plant–pathogen interactions (Kangasjärvi et al., 1994; Sharma and Davis, 1997; Sandermann et al., 1998). This similarity is most likely related to the occurrence of reactive oxygen species (ROS), such as superoxide anion radicals (O2•−) and H2O2 in the apoplast. O3-derived ROS apparently trigger, by way of an as yet undescribed mechanism, an oxidative burst in the affected cells (Schraudner et al., 1998; Pellinen et al., 1999; Rao and Davis, 1999). Similarly, an oxidative burst is one of the earliest responses of plants to microbial pathogens and is an integral component in HR-related cell death (Lamb and Dixon, 1997). Accumulation of H2O2 in response to O3 has been reported in tobacco (Schraudner et al., 1998) and birch (Pellinen et al., 1999). In Arabidopsis, O3-induced accumulation of both O2•− and H2O2 has been reported (Rao and Davis, 1999). Sites of ROS accumulation and visible lesions in these plants were of a distinct size, suggesting that ROS production can function as a regulator of cell death not only in the HR, but also under O3 stress.

O3 is known to activate ethylene and salicylate (SA) signal transduction pathways leading to downstream responses, such as antioxidant and antimicrobial defenses (Sharma et al., 1996; Sharma and Davis, 1997; Sandermann et al., 1998).
1998). Increased ethylene emission from O$_3$-exposed plants is an early, consistent marker for O$_3$ sensitivity (Tingey et al., 1976; Mehlhorn and Wellburn, 1987; Wellburn and Wellburn, 1996; Tuomainen et al., 1997). O$_3$ has been proposed to exert its toxicity through a chemical reaction with ethylene, yielding toxic products that initiate a self-propagating lipid peroxidation cycle (Elstner et al., 1985; Mehlhorn and Wellburn, 1987). Alternatively, ethylene emission has been postulated as a wounding symptom in O$_3$-exposed plants (Heath and Taylor, 1997). However, ethylene has emerged as a regulator of programmed cell death (pcd)—for example, in pea carpel senescence (Orzáez and Granell, 1997), maize endosperm development (Young et al., 1997), and root aerenchyma formation (Drew et al., 2000). Ethylene also triggers pcd in the accelerated cell death1 (acd1) mutant of Arabidopsis (Greenberg and Ausubel, 1993) and is involved in regulating cell death in plant–pathogen interactions (Bent et al., 1992; Ciardi et al., 2000). These results suggest a potential new role for ethylene as a regulator of cell death in O$_3$ responses.

In O$_2$-exposed plants, ethylene synthesis is a result of the specific activation of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase genes (Tuomainen et al., 1997) and is required for O$_3$ damage (Mehlhorn and Wellburn, 1987). Jasmonic acid (JA), on the other hand, can protect tobacco plants from O$_3$ when applied before O$_3$ treatment (Orvar et al., 1997), and the O$_3$ sensitivity of a poplar clone has been proposed to result from JA insensitivity (Koch et al., 1998). Thus, these two signaling pathways, which act synergistically in induced systemic resistance (Pieterse et al., 1998) and in regulating expression of pathogenesis-related (Norman-Setterblad et al., 2000) and wound-induced (O’Donnell et al., 1996) genes, appear to have opposing effects with regard to O$_3$ sensitivity. However, the detailed role or interaction of JA and ethylene signaling in the regulation of ROS accumulation during O$_3$-induced or other forms of ROS-dependent cell death has not been described.

Here, we report the identification and characterization of an Arabidopsis mutant, rcd1 (for radical-induced cell death1), that displays O$_3$-inducible and O$_2$-inducible lesion formation. We also show that O$_3$ response mutants can be useful in identifying interacting components of ROS-dependent signaling pathways in plant–pathogen interactions, for example, to elucidate interaction between ROS and other pathways that regulate the HR lesion formation in incompatible plant–pathogen interactions. Our results suggest that a highly regulated induction of ethylene biosynthesis promotes cell death and that functional ethylene perception and signaling are required for O$_2$* - accumulation, which is responsible for the oxyradical-dependent cell death, ultimately leading to lesion propagation. JA signaling, on the other hand, is involved in lesion containment. On the basis of these results, we propose a model for the relative contribution of the different signaling pathways to ROS-driven lesion propagation and the process of lesion containment.

RESULTS

Isolation and Genetic Mapping of a Novel Arabidopsis Mutant, rcd1, That Displays HR-like Lesion Development in Response to O$_3$

Approximately 14,000 individual M$_2$ plants, grown from ethyl methanesulfonate–mutagenized Columbia (Col-0) seed, were exposed for 3 days to 250 nL L$^{-1}$ O$_3$ (8 hr/day) at a density of 3000 plants m$^{-2}$. Fifty-six individuals displaying O$_3$-induced lesions on rosette leaves were identified. In the subsequent screenings at low density, four lines representing independent loci and distinct patterns and kinetics of lesion formation were selected from 11 lines displaying consistent O$_3$-sensitive phenotypes. When these four lines were crossed with each other and the O$_3$ sensitivity was assayed in the F$_1$ progeny, no O$_3$-sensitive F$_1$ lines were detected, indicating that these mutants represent independent loci. Detailed characterization of the most sensitive mutant, rcd1, is presented here. To determine its mode of inheritance, rcd1 was crossed with Col-0. In the F$_2$ progeny, three phenotypes, both parentals and an intermediate, segregated as a single codominant Mendelian trait, 1:2:1 (36:66:46, $\chi^2 = 3.08$, $P = 0.214$). The F$_2$ progeny from the rcd1 × Landsberg erecta (Ler) were used for mapping with polymerase chain reaction–based microsatellite and cleaved amplified polymorphic sequence markers that cover all five chromosomes. Using 32 homozygous, O$_3$-sensitive rcd1 individuals from the F$_2$ progeny, we were able to position the locus in chromosome 1 at 53 ± 2.2 centimorgans in the recombinant inbred map, 2 centimorgans from a microsatellite in bacterial artificial chromosome clone F23M19. No mutants with similar or related phenotypes had previously been assigned to this region.

O$_3$-induced lesions of rcd1 initiate along the leaf margins and spread inward through intervascular tissue. Normally, visible symptoms appear first as dark, water-soaked spots 12 hr after the onset of O$_3$ treatment (250 nL L$^{-1}$) on middle-aged rosette leaves. When whole tissues are affected by many coalescing lesions, tissue collapse joins medially and progresses toward the leaf base and tip. In this case, damage is already visible at 3 hr as a loss of leaf turgor. By 24 hr, as shown in Figure 1A, lesion progression in rcd1 had halted, and the lesions had developed into well-defined dry patches of dead tissue. Lesion initiation was independent of the duration of O$_3$ exposure (250 nL L$^{-1}$) between 1 and 8 hr; only the extent of lesion formation increased with time. No visible symptoms were found on Col-0 (Figure 1A). However, microscopic examination of Col-0 tissue revealed individual cells and small clusters of cells that had died as a result of O$_3$ exposure. Lesions in rcd1 were not triggered by application of SA (or its active analog benzo[1,2,3]thiadiazole-7-carbothioic acid [BTH]) or ethylene, high-intensity light, UV-B radiation, wounding, or alterations in daylength (data not shown)—all factors known to trigger lesions in other phenotypically related mutants.
Lesion development in rcd1 is transiently comparable with lesion propagation in lsd1 (for lesion simulating disease resistance1), in which extracellular $O_2^{•−}$ production precedes lesion spread (Jabs et al., 1996). This prompted us to assay cellular $O_2^{•−}$ accumulation, as shown in Figure 1B, in $O_3$-exposed rcd1 by monitoring the precipitation of purple formazan when reacting nitro blue tetrazolium (NBT) with $O_2^{•−}$. To exclude reactions of NBT with $O_3$-derived $O_2^{•−}$ (Runeckles and Vaartnou, 1997), the plants were postcultivated in pollutant-free air for at least 15 min after exposure to $O_3$. No NBT precipitation could be detected in clean air-grown rcd1 or Col-0 (data not shown). However, 2 to 4 hr after the onset of $O_3$ exposure, NBT precipitation was visible in both Col-0 and rcd1, although the distribution in each strain differed. In Col-0, NBT precipitation was scattered throughout the leaf, with greater amounts at the tip and margins, whereas in rcd1, staining was concentrated first (at 2 hr) in the region in which the lesions typically initiated and later (at 4 to 8 hr) just beyond the boundary of the collapsing tissue. No NBT staining was observed in Col-0 at 8 hr or later time points. In rcd1, as illustrated in Figure 2, precipitation of NBT by $O_2^{•−}$ continued in front of lesion expansion for as long as 12 hr after the onset of the 6-hr $O_3$ treatment. NBT precipitation clearly was the result of $O_2^{•−}$ accumulation because staining was abolished when superoxide dismutase (SOD) was coinfiltrated into the leaves (Figure 1C).

We used ion leakage, an indicator of plasma membrane damage, as a quantitative measure of the extent of cell death. When ion leakage was monitored in $O_3$-exposed plants, marked differences were found between Col-0 and rcd1 (Figure 3A). In rcd1, ion leakage increased 10-fold during $O_3$ exposure and showed a further increase, even in the absence of $O_3$, between 6 and 12 hr, when the lesions were spreading. In contrast, $O_3$ induced only a small, transient increase in Col-0 during the exposure period.

Extracellular $O_2^{•−}$, but Not $H_2O_2$, Is Both Necessary and Sufficient to Trigger Propagation of Cell Death in rcd1

To elucidate the role of $O_2^{•−}$ and $H_2O_2$ in lesion development, we infiltrated Col-0 and rcd1 leaves with an extracellular $O_2^{•−}$-generating system, xanthine/xanthine oxidase (X/XO); with increasing concentrations of $H_2O_2$; and with an $H_2O_2$-generating system, glucose/glucose oxidase (G/GO) (Jabs et al., 1996; Alvarez et al., 1998). $O_2^{•−}$ production by X/XO lasted for ~3 hr and induced cell death in Col-0 and rcd1 with different magnitudes and kinetics (Figure 3B). In X/XO-infiltrated Col-0 plants, ion leakage increased to ~25% during the first 4 to 8 hr and remained at that value during the experiments. Changes in ion leakage were more dramatic in rcd1, which showed a second increase to as much as 65% between 8 and 24 hr (Figure 3B). Thus, $O_3$ could be
Col-0 leaves with H$_2$O$_2$ solutions up to 10 mM showed no induction of Arabidopsis (Jabs et al., 1996; Alvarez et al., 1998). Infiltration of diphenylene iodonium (DPI), an inhibitor of ROS accumulation in further by inhibition experiments, specifically with diphenylene iodonium (DPI), an inhibitor of ROS accumulation in functionally replaced by O$_2^{•−}$ with X/XO. When the X/XO-produced O$_2^{•−}$ was removed by coinfiltration with MnSOD, cell death in rcd1 was not triggered to any greater extent than in Col-0 (Figure 3C). Therefore, O$_2^{•−}$ alone seems sufficient to induce lesion formation. Physiological concentrations of H$_2$O$_2$ did not induce cell death in either rcd1 or Col-0 in the range of steady state H$_2$O$_2$ production between 5 and 250 µM when compared with buffer alone (data not shown). Furthermore, direct treatment of excised rcd1 or Col-0 leaves with H$_2$O$_2$ solutions up to 10 mM showed no substantial increase in cell death in either strain compared with the buffer control. Greater H$_2$O$_2$ concentrations (50 and 100 mM) resulted in equal increases in cell death in both Col-0 and rcd1 (Figure 3D).

The role of endogenous O$_2^{•−}$ accumulation triggered by O$_3$ during the early phases of lesion formation was studied further by inhibition experiments, specifically with diphenylene iodonium (DPI), an inhibitor of ROS accumulation in Arabidopsis (Jabs et al., 1996; Alvarez et al., 1998). Infiltration of rcd1 leaves with DPI after a 2-hr O$_3$ exposure prevented NBT precipitation (Figure 1C) and reduced leaf damage in a concentration-dependent manner (Figure 4).

Half-maximal reduction was obtained with 2 µM DPI, maximal reduction (~45%) with 5 µM. We conclude from these data that both O$_3$ and O$_2^{•−}$ from X/XO initiate active cellular O$_2^{•−}$ production that continues in rcd1 after exogenous O$_2^{•−}$ ceases. Furthermore, this O$_2^{•−}$ is both necessary and sufficient to propagate cell death in rcd1.

Incompatible Bacterial Pathogen Induces Extensive Cell Death during the HR in rcd1

An oxidative burst, the active production of apoplastic O$_2^{•−}$ and H$_2$O$_2$, is one of the earliest plant responses in incompatible interactions and is integrally involved in regulating hypersensitive cell death (Lamb and Dixon, 1997). To analyze whether incompatible interaction with a bacterial pathogen triggers spreading lesions in rcd1, we infiltrated 3-week-old rosettes of Col-0 and rcd1 with increasing concentrations of Pseudomonas syringae pv tomato, strain DC3000, carrying the avirulence gene avrB on a plasmid (Bent et al., 1992). The extent of tissue collapse caused by the HR was quantified by measuring ion leakage. As seen in Table 1, infiltration of rosette leaves with bacterial concentrations from 10$^4$ to 10$^7$ colony-forming units (cfu) mL$^{-1}$ induced substantially higher ion leakage in rcd1, whereas in mock treatments, no difference between Col-0 and rcd1 was visible. The difference between Col-0 and rcd1 regarding ion leakage was of similar magnitude for every concentration used. Thus, we concluded that spreading lesions are triggered in rcd1 also by an incompatible interaction, which involves ROS production in the oxidative burst.

Oxygen Radicals Induce Pathogen Defense Genes but Not Antioxidant Genes in rcd1 and Col-0

Expression of antioxidant, antimicrobial, and other stress-related genes was analyzed by using 92 expressed sequence tags (ESTs) and cDNA clones in cDNA macroarrays. A subset of the results is shown in Table 2. No major differences were found between Col-0 and rcd1 cultivated in clean air except for lower amounts of catalase (CAT3) and defensin (PDF1.2) mRNAs in rcd1 (Table 2). O$_3$ induced major changes in the expression of PR-1, two glutathione S-transferase genes (GST1 and GST2), and catalases 1 and 3 in both rcd1 and Col-0. O$_2^{•−}$-induced increases in GST1 and GST2 mRNAs were markedly greater in rcd1 (26-fold) than in Col-0 (nine- to 12-fold). CAT3 transcripts increased in both Col-0 and rcd1 such that by 8 hr, they were approximately equivalent. SOD isoforms showed minor responses, or their transcript levels were decreased (chloroplastic Cu/ZnSOD) in both Col-0 and rcd1. We concluded that because the transcript levels for enzymes detoxifying O$_2^{•−}$, H$_2$O$_2$, and lipid hydroperoxides are approximately equal in Col-0 and rcd1, then the radical-induced cell death in rcd1 is not a result of decreased expression of antioxidative genes.
Ethylene Signaling Is Required for Accumulation of O$_2$$^{•–}$, Which Drives Lesion Propagation

Similar concentrations of O$_3$ and O$_2$$^{•–}$ (X/XO treatment) in Col-0 and rcd1 plants led to markedly more cell death in rcd1 (Figures 1B and 2), suggesting that the initial amount of ROS formation from O$_3$ or X/XO is not the only factor involved in the initiation of lesion propagation. Several biochemical variables have been studied as the basis for O$_3$ sensitivity. Ethylene emission has been found to be the most consistent response to O$_3$ (Mehlhorn and Wellburn, 1987; Wellburn and Wellburn, 1996; Tuomainen et al., 1997).

Figure 5 shows results of detailed studies on ethylene biosynthesis in Col-0 and rcd1. Of the five Arabidopsis ACC synthase genes analyzed—AT-ACS1, 2, 4, 5, and 6—only the isoform AT-ACS6 was responsive to O$_3$. AT-ACS6 was already highly induced in rcd1 at 30 min after the onset of O$_3$ exposure (Figure 5A). Transcript levels were at their maximum at 1 to 2 hr and decreased thereafter. Enhanced activation of AT-ACS6 in rcd1 was also reflected in increased ACC concentrations (Figure 5B). Similarly, the amounts of ACC oxidase transcripts (Table 2) and ethylene evolution (Figure 5C) were also three- to fivefold higher in rcd1 than in Col-0, and in contrast to Col-0, ethylene evolution in rcd1 continued past the period of O$_3$ exposure.

It has been proposed (Elstner et al., 1985; Mehlhorn and Wellburn, 1987) that a chemical reaction between ethylene and O$_3$ could produce water-soluble, highly reactive radicals that could initiate a self-propagating peroxidative cycle, thus causing ethylene-mediated damage without the action of ethylene signaling. Results of the experiments addressing this question are shown in Figure 6. rcd1 was exposed first to O$_3$, followed by incubation either in clean air or in the presence of an antagonist of ethylene action, norbornadiene (the black bar) and postcultivated in pollutant-free air. The extent of cell death was measured as relative ion leakage (percentage of total ions ±SE).

(A) O$_3$-induced cell death. Leaves from clean air-grown plants were detached and vacuum-infiltrated with the O$_2$$^{•–}$-generating system xanthine and xanthine oxidase (X/XO). The black triangle under the x axis indicates the approximate duration and intensity of O$_2$$^{•–}$ synthesis by X/XO. The time course of cell death, measured as relative ion leakage (±SE), was monitored for 24 hr.

(B) O$_2$$^{•–}$-induced cell death. Leaves from clean air-grown plants were detached and vacuum-infiltrated with increasing concentrations of H$_2$O$_2$. Cell death in Col-0 and rcd1 was measured as relative ion leakage (±SE) after 20 hr.

(C) Inhibition of cell death by SOD. Leaves of Col-0 and rcd1 were infiltrated with the O$_2$$^{•–}$-generating system X/XO. MnSOD (440 units mL$^{-1}$), or infiltration buffer, as indicated. Reagents were included (+) or not (−) in each treatment as indicated. Cell death was measured as ion leakage (±SE) after 20 hr.

(D) H$_2$O$_2$ and cell death. Leaves from clean air-grown plants were detached and vacuum-infiltrated with increasing concentrations of H$_2$O$_2$. Cell death in Col-0 and rcd1 was measured as relative ion leakage (±SE) after 20 hr.

**Figure 3.** Progression of O$_3$-Induced and O$_2$$^{•–}$-Induced Leaf Damage in Col-0 and rcd1.

(A) O$_3$-induced cell death. Plants were exposed to O$_3$ (indicated by the black bar) and postcultivated in pollutant-free air. The extent of cell death was measured as relative ion leakage (percentage of total ions ±SE).

(B) O$_2$$^{•–}$-induced cell death. Leaves from clean air-grown plants were detached and vacuum-infiltrated with the O$_2$$^{•–}$-generating system xanthine and xanthine oxidase (X/XO). The black triangle under the x axis indicates the approximate duration and intensity of O$_2$$^{•–}$ synthesis by X/XO. The time course of cell death, measured as relative ion leakage (±SE), was monitored for 24 hr.

(C) Inhibition of cell death by SOD. Leaves of Col-0 and rcd1 were infiltrated with the O$_2$$^{•–}$-generating system X/XO. MnSOD (440 units mL$^{-1}$), or infiltration buffer, as indicated. Reagents were included (+) or not (−) in each treatment as indicated. Cell death was measured as ion leakage (±SE) after 20 hr.

(D) H$_2$O$_2$ and cell death. Leaves from clean air-grown plants were detached and vacuum-infiltrated with increasing concentrations of H$_2$O$_2$. Cell death in Col-0 and rcd1 was measured as relative ion leakage (±SE) after 20 hr.
The role of ethylene signaling in O₃-induced tissue damage was additionally addressed with the ethylene-insensitive mutant ein2 (Guzman and Ecker, 1990). After exposure to 250 nL L⁻¹ O₃ for 4 hr, rcd1 displayed more cell death than did Col-0 and ein2 (Figure 6B). When O₃ was followed by 4 hr of ethylene (12 ± 2 μL L⁻¹), a marked increase in cell death was evident in both rcd1 and Col-0 but not in ein2. Ethylene treatment alone did not induce O₂•− accumulation or lesion formation in rcd1 (not shown). Furthermore, when the plants were exposed to 400 nL L⁻¹ O₃, leaf damage in both Col-0 and rcd1 was extensive (Figure 6B), and predominant accumulation of O₂•− was apparent in Col-0 (Figure 1C). Under the same conditions, ein2 showed just minor O₂•− accumulation at 2 hr (Figure 1C) and no later leaf damage. Enhanced ethylene synthesis (by two- to fourfold), however, was evident in both Col-0 and ein2 under this regime (Figure 6B, insert), indicating that O₃ enters the leaves and elicits a response in both strains. To address whether O₂•− in combination with endogenously produced ethylene has similar effects as O₃ and exogenous ethylene, leaves of Col-0, rcd1 and ein2 were infiltrated with X/XO in the presence and absence of the ethylene precursor ACC (Figure 6C). X/XO-induced cell death, determined as ion leakage after 20 hr, increased in Col-0 (by twofold), rcd1 (by threefold), and ein2 (by fivefold) in comparison with buffer controls. Although ACC alone had a negligible effect, O₂•− and ACC together had synergistic effects over O₂•− alone in Col-0 (3.5-fold increase) and rcd1 (4.5-fold increase) but not in ein2.

The position of rcd1 relative to the ethylene pathway was further elucidated by crossing rcd1 with ein2 and identifying the F₂ individuals that were homozygous for both mutations. Epistatic relationships between the loci were analyzed by exposing the double mutant to 4 hr of O₃ followed by 4 hr of clean air in a setup similar to that used with NBD. Figure 7 shows that no differences were detected in ion leakage in O₃-exposed Col-0 or ein2 during the course of the experiments. In O₃-exposed rcd1, ion leakage increased from control values to 9% leakage during the 4-hr O₃ exposure and continued to increase to 16% during the subsequent 4 hr in clean air. The rcd1 ein2 double mutant showed an increase in ion leakage during the 4-hr O₃ exposure similar to that for rcd1. However, the spread of cell death in clean air after O₃ was absent, and by 8 hr, ion leakage had decreased back to the control value (Figure 7). Ethylene insensitivity in the double mutant thus prevented the occurrence of spreading cell death after O₃ exposure in a manner similar to NBD treatment (Figure 6A) without affecting the increase in cell death during O₃ exposure. Thus, we concluded that both a highly regulated increase in ethylene evolution and functional ethylene signaling are required for the amplification of cell death.

Ethylene Insensitivity Confers O₃ Tolerance

The role of ethylene signaling in O₃-induced tissue damage was additionally addressed with the ethylene-insensitive mutant ein2 (Guzman and Ecker, 1990). After exposure to 250 nL L⁻¹ O₃ for 4 hr, rcd1 displayed more cell death than did Col-0 and ein2 (Figure 6B). When O₃ was followed by 4 hr of ethylene (12 ± 2 μL L⁻¹), a marked increase in cell death was evident in both rcd1 and Col-0 but not in ein2. Ethylene treatment alone did not induce O₂•− accumulation or lesion formation in rcd1 (not shown). Furthermore, when the plants were exposed to 400 nL L⁻¹ O₃, leaf damage in both Col-0 and rcd1 was extensive (Figure 6B), and predominant accumulation of O₂•− was apparent in Col-0 (Figure 1C). Under the same conditions, ein2 showed just minor O₂•− accumulation at 2 hr (Figure 1C) and no later leaf damage. Enhanced ethylene synthesis (by two- to fourfold), however, was evident in both Col-0 and ein2 under this regime (Figure 6B, insert), indicating that O₃ enters the leaves and elicits a response in both strains. To address whether O₂•− in combination with endogenously produced ethylene has similar effects as O₃ and exogenous ethylene, leaves of Col-0, rcd1 and ein2 were infiltrated with X/XO in the presence and absence of the ethylene precursor ACC (Figure 6C). X/XO-induced cell death, determined as ion leakage after 20 hr, increased in Col-0 (by twofold), rcd1 (by threefold), and ein2 (by fivefold) in comparison with buffer controls. Although ACC alone had a negligible effect, O₂•− and ACC together had synergistic effects over O₂•− alone in Col-0 (3.5-fold increase) and rcd1 (4.5-fold increase) but not in ein2.

The position of rcd1 relative to the ethylene pathway was further elucidated by crossing rcd1 with ein2 and identifying the F₂ individuals that were homozygous for both mutations. Epistatic relationships between the loci were analyzed by exposing the double mutant to 4 hr of O₃ followed by 4 hr of clean air in a setup similar to that used with NBD. Figure 7 shows that no differences were detected in ion leakage in O₃-exposed Col-0 or ein2 during the course of the experiments. In O₃-exposed rcd1, ion leakage increased from control values to 9% leakage during the 4-hr O₃ exposure and continued to increase to 16% during the subsequent 4 hr in clean air. The rcd1 ein2 double mutant showed an increase in ion leakage during the 4-hr O₃ exposure similar to that for rcd1. However, the spread of cell death in clean air after O₃ was absent, and by 8 hr, ion leakage had decreased back to the control value (Figure 7). Ethylene insensitivity in the double mutant thus prevented the occurrence of spreading cell death after O₃ exposure in a manner similar to NBD treatment (Figure 6A) without affecting the increase in cell death during O₃ exposure. Thus, we concluded that both a highly regulated increase in ethylene evolution and functional ethylene signaling are required for the amplification of cell death.

**Table 1. Avirulent Pathogen Triggers Spreading Cell Death in rcd1**

<table>
<thead>
<tr>
<th>cfu/mL</th>
<th>Col-0</th>
<th>rcd1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>9.4 ± 0.5 ab</td>
<td>9.2 ± 0.7 a</td>
</tr>
<tr>
<td>10⁶</td>
<td>12.6 ± 0.5 ab</td>
<td>20.9 ± 2.5 bc</td>
</tr>
<tr>
<td>10⁸</td>
<td>17.4 ± 1.3 bc</td>
<td>25.5 ± 2.4 c</td>
</tr>
<tr>
<td>10⁹</td>
<td>41.1 ± 3.3 d</td>
<td>53.2 ± 1.7 e</td>
</tr>
<tr>
<td>10⁵</td>
<td>59.9 ± 3.3 e</td>
<td>71.9 ± 3.0 f</td>
</tr>
</tbody>
</table>

*Cell death is measured as relative ion leakage (percentage of total ions) from the rosette leaves of Col-0 and rcd1 at 20 hr after infiltration with varying concentrations of the avirulent bacterial pathogen P. s. tomato DC3000 carrying the avirulence gene avrB in a plasmid.

* Means (±SE, n = 5) followed by the same letter are not significantly (P < 0.05) different according to Tukey’s multiple range test.
looked at other possible signaling pathways that could be involved in lesion containment. Kangasjärvi et al. (1994) proposed that JA could act as signaling molecule in plant–O3 interactions. Furthermore, Table 2 shows that fewer transcripts of defensin (PDF1.2), a gene that is coregulated by ethylene and JA (Penninckx et al., 1998), were present in both clean air–grown and O3-exposed rcd1. This suggests that JA biosynthesis or signaling, or interaction of JA and ethylene signaling, could be affected in rcd1, because the ethylene biosynthesis and signaling pathways in rcd1 are functional.

Results of the experiments addressing the role of JA in lesion containment are shown in Figure 8. rcd1 was first treated with methyl jasmonate (MeJA) after 4 hr of O3 exposure in an experiment similar to that used with the ethylene antagonist NBD (cf. with Figure 6A). Exposure of rcd1 to 1.4 μM MeJA after O3 exposure inhibited lesion propagation from 4 to 8 hr (Figure 8A). This suggests that JA treatment is counteracting or preventing the lesion propagation executed by the ethylene-dependent O2•− accumulation in rcd1. Furthermore, this suggests that rcd1 is not JA insensitive. Table 3 shows further verification: Roots of rcd1 were sensitive to growth inhibition by MeJA, whereas JA-insensitive jar1 was not affected. In the presence of 10 μM MeJA, root growth inhibition in rcd1 was similar to that in Col-0 (61 and 66%, respectively), whereas in jar1, no substantial inhibition occurred. Thus, based on JA inhibition of

### Table 2. Stress and Defense Gene Transcript Levels

<table>
<thead>
<tr>
<th>Gene</th>
<th>EST Stock/Accession No.</th>
<th>Air Col-0</th>
<th>rcd1 Col-0</th>
<th>Air rcd1 Col-0</th>
<th>Air rcd1 rcd1</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR1</td>
<td>M90508</td>
<td>0.1c</td>
<td>0.1</td>
<td>2.0d</td>
<td>2.3f</td>
</tr>
<tr>
<td>CHlb</td>
<td>92G1T7</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>GST1</td>
<td>206N21T7</td>
<td>0.4</td>
<td>0.1</td>
<td>5.0f</td>
<td>11f</td>
</tr>
<tr>
<td>GST2</td>
<td>91H22T7</td>
<td>0.6</td>
<td>0.5</td>
<td>6.5f</td>
<td>13f</td>
</tr>
<tr>
<td>AT-ACO2</td>
<td>G2B6T7</td>
<td>0.3</td>
<td>0.5</td>
<td>0.6</td>
<td>1.6f</td>
</tr>
<tr>
<td>MNSOD1</td>
<td>109J19T7</td>
<td>1.6</td>
<td>2.3</td>
<td>2.0</td>
<td>1.6</td>
</tr>
<tr>
<td>Cu/ZnSOD1</td>
<td>2G11T7</td>
<td>2.5</td>
<td>3.3</td>
<td>3.0</td>
<td>4.2</td>
</tr>
<tr>
<td>Cu/ZnSOD2</td>
<td>161I22T7</td>
<td>4.5</td>
<td>5.1</td>
<td>2.6</td>
<td>1.6</td>
</tr>
<tr>
<td>FeSOD1</td>
<td>34D9T7</td>
<td>1.0</td>
<td>2.2</td>
<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>APX1</td>
<td>135D24T7</td>
<td>1.9</td>
<td>2.2</td>
<td>1.7</td>
<td>2.7</td>
</tr>
<tr>
<td>GR</td>
<td>185P3T7</td>
<td>1.1</td>
<td>1.7</td>
<td>0.3</td>
<td>0.8</td>
</tr>
<tr>
<td>GPX2</td>
<td>190H7T7</td>
<td>2.0</td>
<td>2.2</td>
<td>0.7</td>
<td>1.9</td>
</tr>
<tr>
<td>catalase3</td>
<td>134I1T7</td>
<td>5.3</td>
<td>2.2</td>
<td>12f</td>
<td>10f</td>
</tr>
<tr>
<td>catalase1</td>
<td>118M15T7</td>
<td>0.5</td>
<td>0.3</td>
<td>2.2f</td>
<td>1.0f</td>
</tr>
<tr>
<td>AOS</td>
<td>230J8T7</td>
<td>0.8</td>
<td>0.6</td>
<td>0.5</td>
<td>0.3</td>
</tr>
<tr>
<td>PDF1.2</td>
<td>37F10T7</td>
<td>1.3</td>
<td>0.4</td>
<td>1.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

a Expression of selected stress- and defense-related genes in wild-type Col-0 and the rcd1 mutant in ambient air and at 8 hr after the beginning of a 6-hr O3 exposure (250 nL L−1). A complete list of the genes used can be seen at [http://www.biocenter.helsinki.fi/bi/koivu/ Arabidopsis/arrays.html](http://www.biocenter.helsinki.fi/bi/koivu/ Arabidopsis/arrays.html).

b PR, pathogenesis-related protein; CHlb, basic chitinase; GST, glutathione S-transferase; ACO, ACC oxidase; SOD, superoxide dismutase; APX, ascorbate peroxidase; GR, glutathione reductase; GPX, glutathione peroxidase; AOS, allene oxide synthase; PDF, plant defensin.

c The values depict mRNA abundance relative to the mean of mRNA abundance of actin genes ACT2 (EST accession number 179M16T7) and ACT8 (accession number 179P1T7), which were shown to be constitutively expressed by RNA gel blots.

d †, induction two- to fourfold; ‡, induction fivefold and above. Similar results have been seen using mRNA from three independent experiments.

### Figure 5. O3 Induction of Ethylene Biosynthesis.

(A) Time course of O3 induction of the ACC synthase gene AT-ACs6. Transcript levels shown are relative to the hybridization signal of control samples taken at the same times.

(B) Time course of concentrations of the ethylene precursor ACC (±SE).

(C) Ethylene evolution (±SE) in clean air–grown and O3-exposed Col-0 and rcd1. O3 exposure (250 nL L−1 for 6 hr) was followed by postcultivation in pollutant-free air. Black bars indicate the duration of exposure. FW, fresh weight.
root elongation, rcd1 cannot be regarded as JA insensitive. rcd1 is not deficient in JA biosynthesis either, because exposure of rcd1 to O₃ led to much greater concentrations of JA than in Col-0 (not shown).

To elucidate the role of JA in lesion propagation and containment, we further utilized the JA-insensitive mutant jar1 (Staswick and Howell, 1992). O₃-exposed jar1 developed spreading lesions similar to those of rcd1 (Figure 8B). Furthermore, in the margins of the spreading lesion, jar1 exhibited O₂•− accumulation (Figure 8D) in a manner similar to rcd1. The same initial O₂•− accumulation as in Col-0 at 1 to 2 hr seemed sufficient to initiate lesion propagation in jar1. However, the O₃-induced lesion propagation in jar1 was not accompanied by similar high amounts of ethylene as were seen in rcd1 (Figure 8E). This suggests that jar1 is less efficient in lesion containment, in contrast to rcd1, which shows increased lesion propagation. Thus, these results suggest that JA signaling is required in processes that prevent the O₂•−-dependent lesion propagation or that contain lesion spread.

**DISCUSSION**

The novel rcd1 mutant described here is sensitive to extracellular oxygen radicals and shows extensive formation of HR-like lesions and activation of several pathogen defense-related processes in response to ROS. Phenotypically, rcd1 is in part similar to the acd (Greenberg and Ausubel, 1993) and lsd (Dietrich et al., 1994) mutants that have been utilized to identify the components regulating hypersensitive cell death downstream of pathogen infection. In particular, the “runaway” spread of cell death in rcd1 during the first 8 to 12 hr is reminiscent of that in lsd1, in that a front of O₂•− accumulation has been found at lesion boundaries in living tissues in both mutants. However, in addition to avirulent *Pseudomonas*, which induces an oxidative burst in the affected cells, O₂•− and O₃ are the only treatments known thus far to trigger the spreading lesion formation in rcd1, whereas various other factors, such as SA, BTH, ethylene, or growth conditions, trigger lesion formation in *lsd* or *acd* mutants. Thus, rcd1 seems to define a component of the HR related to the role of ROS in cell death regulation. The chromosomal location of rcd1, in addition, has not been described for any *acd*, *lsd*, antioxidant, or disease-related mutants. Therefore, rcd1 describes a novel class of ROS-responsive lesion-mimic mutants.

**rcd1 Appears to Be Deficient in the Control of Lesion Propagation**

The phenotypes described in various lesion-mimic mutants suggest that three separate processes—initiation, propagation,
Increased \(O_3\) and \(O_2^{=}\)-Induced Lesion Propagation in \(rcd1\) Is Not a Result of Deficient Antioxidative Protection

Propagation of the lesion is most likely to involve generation at the initiation site of a signal that regulates death of the neighboring cells. Both \(O_2^{=}\) and \(H_2O_2\) have been shown to act as signal molecules in various systems (Lamb and Dixon, 1997). \(O_3\)-exposed tobacco (Schraudner et al., 1998) and birch (Pellinen et al., 1999) showed predominant \(H_2O_2\) but no \(O_2^{=}\) accumulation, whereas \(O_3\)-exposed Arabidop-

sia predominately accumulates \(O_2^{=}\). Our results suggest that in Arabidopsis, \(O_2^{=}\) can act as a positive regulator in the amplification of a cellular oxidative burst and cell death in the surrounding cells. \(O_3\) and \(O_2^{=}\) generated by X/XO, induced \(O_2^{=}\) accumulation and cell death in \(rcd1\) (Figure 1B), whereas \(H_2O_2\) concentrations as great as 10 mM were ineffective (Figure 3D). This raises the question of the functionality of \(O_2^{=}\) scavenging in \(rcd1\). LSD1 is known to be involved in the induction of CuZnSOD in Arabidopsis (Kliebenstein et al., 1999), and \(O_3\) sensitivity of \(vtc1\) is a result of low ascorbate concentrations (Conklin et al., 1996). Similarly, several \(O_3\)-sensitive mutants that are deficient in Fe and CuZnSOD gene expression have been isolated (D. Kliebenstein and R. Last, unpublished data). However, transcript levels of several antioxidant genes were approximately the same in \(rcd1\) and Col-0 at 8 hr after the beginning of the exposure (Table 2). Furthermore, ascorbate concentrations and total catalase and SOD activities were similar in both clean air–grown and \(O_3\)-exposed Col-0 and \(rcd1\) (K. Overmyer, H. Tuominen, and J. Kangasjärvi, unpublished data). Thus, at an overall level, deficient antioxidant capacity does not seem to be responsible for lesion propagation in \(rcd1\); other signals and mechanisms must be involved.

**Specific Activation of Ethylene Biosynthesis and Functional Ethylene Signaling Are Required for Amplification of \(O_2^{=}\) Accumulation and Cell Death**

Ethylene synthesis in the \(O_3\)-exposed \(rcd1\) was a result of fast, specific activation of the ACC synthase gene \(AT\text{-}ACS6\) (Figure 5A). A similar \(O_3\) responsiveness of only the \(AT\text{-}ACS6\) gene has also been seen in Col-0 (Vahala et al., 1998). In Col-0, both \(O_3\)-induced ethylene evolution and \(O_2^{=}\) accumulation lasted approximately the same time as the \(O_3\) exposure, whereas in \(rcd1\) they continued beyond the actual exposure period (Figures 1B and 5). Similarly, a correlation between ethylene evolution and \(O_2^{=}\) accumulation was evident in a survey of 11 Arabidopsis ecotypes differing in their sensitivity to \(O_3\) (K. Mittelstrass, H. Wohlgemuth, and C. Langebartels, unpublished data). A direct role for ethylene in regulating ROS production was shown in further experiments. Both loss-of-function and gain-of-function experiments with \(O_3\)-sensitive \(rcd1\) and \(O_3\)-tolerant Col-0 and \(ein2\) revealed a requirement for ethylene synthesis and signaling in cell death. This suggests that the \(O_3\)-induced ethylene synthesis in Arabidopsis is not a mere indicator of damage (Tingey et al., 1976; Heath and Taylor, 1997) but rather an active component controlling the damage process itself. It also implies that ethylene acts upstream of \(O_2^{=}\) accumulation, which is consistent with its rapid induction by \(O_3\). However, both 250 and 400 nL L\(^{-1}\) \(O_3\) induced ethylene evolution and initial microbursts of \(O_2^{=}\) in \(ein2\) without lesion propagation. This suggests that the increase in ethylene synthesis is not under autocatalytic regulation and that these very early responses related to lesion initiation are...
The experiments performed with the rcd1 ein2 double mutant also separated functionally O$_3$-induced lesion initiation from propagation. The double mutant showed an initial increase in cell death during O$_3$ exposure, a property of rcd1, but ceased further lesion propagation in clean air because of the ethylene insensitivity conferred by ein2 (Figure 7). Together, these results suggest that ethylene-independent lesion initiation is followed by ethylene-dependent amplification of O$_2^{•-}$ accumulation, which is responsible for the execution of spreading cell death. We propose that ethylene primes the cells in the borders of the lesions for hypersensitivity to a message from the O$_2^{•-}$-producing cells, which results in triggering O$_2^{•-}$ production or in enhanced O$_2^{•-}$ production.

JAs and JA Signaling Are Involved in Lesion Containment

JA signaling appears to be involved in lesion containment. Exposure of rcd1 to MeJA after 4 hr of O$_3$ prevented further lesion propagation (Figure 8A). This suggests that increased JA can override the ethylene and O$_2^{•-}$-dependent propagation of cell death that occurs in rcd1 at that time. Further evidence for this comes from the fact that the JA-insensitive jar1 is O$_3$ sensitive and displays a spreading-lesion phenotype similar to that of rcd1. Initially, at 1 to 2 hr after the beginning of O$_3$ exposure, the pattern of O$_2^{•-}$ accumulation in jar1 was similar to that of Col-0 and rcd1. Later, during lesion propagation, O$_2^{•-}$ accumulation continued in jar1, but in contrast to rcd1, propagation did not involve ethylene evolution to the same extent as in rcd1. This may seem at first contradictory to the proposed requirement for ethylene as an amplifier of O$_2^{•-}$ accumulation and cell death. Actually, however, similar lesion phenotypes would be expected to result from an increase in lesion propagation caused by increased ethylene-dependent O$_2^{•-}$ accumulation, as in rcd1, or from a reduced capacity for lesion containment, without a large increase in ethylene, as in jar1.

Does Interaction and Cross-Talk among JA, SA, and Ethylene Signaling Determine the Degree and Extent of O$_2^{•-}$-Driven Lesion Formation?

Recently, an oxidative cell death cycle was postulated from work with pathogen-infected plants undergoing HR (Van Camp et al., 1998). In this model, ROS, SA, and cell death were implicated in a self-amplifying cycle ultimately leading to visible symptom development. Rao and Davis (1999) have shown that NahG Arabidopsis plants failed to develop HR-like lesions after a short exposure to O$_3$. They concluded that SA is involved in processes that contribute to O$_3$-induced cell death by potentiating ROS toxicity. We have also verified this in our own experiments (H. Tuominen, K.
ethylene biosynthesis was activated in respect to processes that regulate cell death (Figure 9). Ethylene, appear to interact with ROS in HR-like lesion development that takes place in O3-exposed Arabidopsis. Their interactions and roles in ROS-dependent cell death are summarized in Figure 9. During lesion initiation, initial cellular O2•− accumulation in microbursts executes cell death in separate, individual cells, resulting in micro-HR-like microscopic lesions. SA is required for the HR-like cell death, because O3 does not induce HR-like lesions in NahG plants (Rao and Davis, 1999). Activation of the ACC synthase gene AT-ACS6 in rcd1 results in increased ethylene evolution, which promotes the ethylene signaling–dependent O2•− accumulation that drives lesion propagation. A similar role for ethylene was also postulated for the activation of cell death in the acd6 mutant (Rate et al., 1999) and for regulating symptom development in Pseudomonas- and Xanthomonas-infected Arabidopsis (Bent et al., 1992) and in Xanthomonas-infected tomato (Ciardi et al., 2000).

Exogenously added JA promoted lesion containment in rcd1, also overriding the positive effect of ethylene on cell death. Furthermore, the JA-insensitive jar1 showed a lack of lesion containment and thus formed visible lesions and O2•− accumulation without high ethylene synthesis. A similar role for JA was proposed in an O3-sensitive poplar clone (Koch et al., 1998).

Results from the experiments presented here give some indication as to the possible location of RCD1 function with respect to processes that regulate cell death (Figure 9). Ethylene biosynthesis was activated in rcd1 to a higher degree, or by a lesser stimulus than in the parent ecotype Col-0. RCD1 may be involved in processes related to regulation of ethylene biosynthesis or to communication between the ethylene, JA, and SA pathways, which are known to interact with each other (Reymond and Farmer, 1998). Interaction between these pathways appears to fine-tune the relative contribution of lesion initiation, propagation, and containment, as reflected by different lesion sizes and formation kinetics. It therefore seems obvious to include both ethylene and JA, as presented in Figure 9, as new regulating components in the oxidative cell death cycle.

| Table 3. Inhibition of Root Elongation by MeJa in Col-0, rcd1, and jar1 |
|-----------------------------|--------|--------|--------|
| MeJa (μM) | Col-0 | rcd1 | jar1 |
| 0 | 34.4 ± 1.6 | 26.6 ± 0.7 b | 28.3 ± 1.0 b |
| 10 | 11.6 ± 1.1 a | 10.2 ± 0.4 a | 26.4 ± 0.7 b |

a Seeds were germinated and grown on vertically placed agar plates containing 0 or 10 μM MeJa.

| RESULTS FROM THE EXPERIMENTS PRESENTED HERE GIVE SOME INDICATION AS TO THE POSSIBLE LOCATION OF RCD1 FUNCTION WITH RESPECT TO PROCESSES THAT REGULATE CELL DEATH (FIGURE 9). ETHYLENE BIOSYNTHESIS WAS ACTIVATED IN RCD1 TO A HIGHER DEGREE, OR BY A LESSER STIMULUS THAN IN THE PARENT ECOTYPE COL-0. RCD1 MAY BE INVOLVED IN PROCESSES RELATED TO REGULATION OF ETHYLENE BIOSYNTHESIS OR TO COMMUNICATION BETWEEN THE ETHYLENE, JA, AND SA PATHWAYS, WHICH ARE KNOWN TO INTERACT WITH EACH OTHER (REYMOND AND FARMER, 1998). INTERACTION BETWEEN THESE PATHWAYS APPEARS TO FINE-TUNE THE RELATIVE CONTRIBUTION OF LESION INITIATION, PROPAGATION, AND CONTAINMENT, AS REFLECTED BY DIFFERENT LESION SIZES AND FORMATION KINETICS. IT THEREFORE SEEMS OBVIOUS TO INCLUDE BOTH ETHYLENE AND JA, AS PRESENTED IN FIGURE 9, AS NEW REGULATING COMPONENTS IN THE OXIDATIVE CELL DEATH CYCLE. |

METHODS

Mutant Screening, Crossing, and Genetic Mapping

Ethyl methanesulfonate–mutagenized Arabidopsis thaliana ecotype Columbia (Col-0) seeds (M2E-1A-4; Lehle Seeds, Round Rock, TX) were grown in a peat/sand mixture at a density of 3000 plants m−2 for 3 weeks in growth chambers (photon flux density 250 μmol m−2 sec−1, 12/12 hr day/night, 22/16°C, 50/75% relative humidity) and exposed for 3 days to 250 nL L−1 ozone (O3) for 8 hr. Details of the screening procedure for O3-induced foliar lesions have been described (Langebartels et al., 2000). Selected mutants were crossed with each other as appropriate to determine allelism with Col-0 to determine the inheritance of the phenotype, and with Landsberg erecta (Ler) for mapping. F2 populations of rcd1 × Ler with an O3-sensitive phenotype were used for linkage analysis by using cleaved amplified polymorphic sequence and microsatellite markers. O3 sensitivity of the F2 lines used was verified in selfed F2 populations. The ethylene-insensitive ein2-1 mutant and the jasmonic acid (JA)-insensitive jar1-1 were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus). For double mutant analysis, ethylene-insensitive F2 individual from the cross rcd1 × ein2-1 were selected on plates containing 20 μM 1-aminoacyclopropane-1-carboxylic acid (ACC) by screening for the lack of triple response. Double mutant F2 individual homozygous for rcd1 were identified and
confirmed with backcrosses of ethylene-insensitive F₂ and F₃ plants to rcd1.

**Treatments and Biochemical Analyses**

O₃ exposures were a single 6-hr pulse at 250 nL L⁻¹, except where otherwise indicated. Times of measurement refer to hours after the onset of exposure. Superoxide (O₂⁻) accumulation in leaves was visualized with 0.1% nitroblue tetrazolium (NBT; Boehringer Mannheim), as described (Jabs et al., 1996), with a 20-min incubation period. Plants were removed from the chambers at least 15 min before staining. Ethylene exposures (12 ± 2 μL L⁻¹) were performed under conditions similar to those described above for O₂ by mixing 2% ethylene with the air in the growth chamber. The concentration of ethylene was measured with a photoionizer and regulated with a computer-controlled system. Exposures to high-intensity light (photon flux density 1000 μmol m⁻² sec⁻¹) supplied by Philips multimetal lamps; Oy Philips AB, Espoo, Finland) were for 8 hr in growth chambers at 10°C. UV-B exposure (0.3 kJ m⁻² hr⁻¹) was for 7 days and was essentially as described (Landry et al., 1995). Treatments with 30 μL L⁻¹ ethylene antagonist norbornadiene (NBD; Sigma) and 1.4 μM methyl jasmonate (MeJA; Sigma) were performed in a desiccator jar. Ethylene emission from plants was analyzed from two rosette leaves into 5 mL of 18 Mohm water for 1 hr, measured replicates per treatment. Cell death was quantified by ion leakage by microscopic examination of roots and hypocotyls. Ethylene exposures (12 ± 2 μL L⁻¹) were performed under conditions similar to those described above for O₂ by mixing 2% ethylene with the air in the growth chamber. The concentration of ethylene was measured with a photoionizer and regulated with a computer-controlled system. Exposures to high-intensity light (photon flux density 1000 μmol m⁻² sec⁻¹) supplied by Philips multimetal lamps; Oy Philips AB, Espoo, Finland) were for 8 hr in growth chambers at 10°C. UV-B exposure (0.3 kJ m⁻² hr⁻¹) was for 7 days and was essentially as described (Landry et al., 1995). Treatments with 30 μL L⁻¹ ethylene antagonist norbornadiene (NBD; Sigma) and 1.4 μM methyl jasmonate (MeJA; Sigma) were performed in a desiccator jar. Ethylene emission from plants was analyzed from two rosette leaves into 5 mL of 18 Mohm water for 1 hr, measured replicates per treatment. Cell death was quantified by ion leakage.

**In Vitro Treatments**

The O₂⁻-generating system xanthine/xanthine oxidase (X/XO; 0.5 mM/0.05 unit mL⁻¹; Sigma) or the H₂O₂-generating system glucose/glucose oxidase (G/GO; 2.5 mM/2.5 to 250 units mL⁻¹; Calbiochem) in 10 mM sodium phosphate buffer, pH 7.0, was vacuum-infiltrated into detached leaves (Jabs et al., 1996; Alvarez et al., 1998). When indicated, 440 units mL⁻¹ MnSOD (Sigma) or 50 μM ACC (Sigma) was included. All treatments were for 20 hr (or as indicated) at 22°C, and only the first three fully expanded leaves were used. Treatment concentrations in leaves in each treatment were assessed by NBT staining. H₂O₂ production was monitored and calibrated against standard dilutions by luminol bioluminescence for concentrations in the micromolar range and by direct spectrophotometric (A₄₅₀) measurement for concentrations in the millimolar range, as described (Chamnongpol et al., 1998). Direct H₂O₂ treatments were performed in a large volume to prevent H₂O₂ clearance by detoxification systems (50 mL per 10 leaves); concentration determinations before and after treatments indicated a maximum of 20% decrease in H₂O₂ over the course of all experiments. For JA root inhibition assays, surface-sterilized seed was grown on Murashige and Skoog (1962) medium in the presence or absence of 10 μM MeJA for 7 days, after which root length was determined as described by Staswick and Howell (1992). Pathogen treatments were performed with detached rosettes that were maintained on wet filter paper during the course of the treatment. Freshly grown Pseudomonas syringae pv tomato DC3000 bacteria bearing the AvrB gene on the plasmid pPSG0002 (Bent et al., 1992) were resuspended, diluted at the indicated densities in 10 mM MgCl₂, and delivered by vacuum infiltration. All experiments were repeated at least three times.

**RNA Isolation, RNA Gel Blot, and cDNA Macroarray Hybridizations**

RNA isolation and hybridizations were essentially as described (Carpenter and Simon, 1998). The expression of 92 selected genes was studied with cDNA macroarrays using cDNA clones and with expressed sequence tag clones from the Arabidopsis Biological Resource Center. Inserts were amplified by polymerase chain reaction, purified, and examined by agarose gel electrophoresis. Amplified polymerase chain reaction products (100 ng) were denatured and blotted onto a Hybond N+ membrane (Amersham) by using a 96-well dot-blot device and then cross-linked with UV light. Used as negative controls were 100 ng of pSPORT vector and 100 ng of oligo-dT₂₁. ³²P-labeled cDNA probes were prepared from 1 μg of mRNA by oligo-dT₂₁–primed polymerization with M-MLV reverse transcriptase (Promega) at 42°C for 1 hr, followed by 15 min at 70°C, and then rapid cooling on ice. After addition of 1.5 units of RNase H (Stratagene), the mixture was incubated at 37°C for 30 min and purified on G-50 columns (Amersham). Hybridizations were at 42°C in a 50% formamide buffer with stringent washes at 65°C (0.2 × SSC [1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate] and 0.1% SDS), according to standard protocols. All hybridizations were quantified with a phosphor imager (Bas-1500; FujiFilm, Tokyo, Japan) and an image analysis program.

**ACKNOWLEDGMENTS**

We thank Mr. Timo Oksanen for help with the O₃ exposures during the mutant screening, Mr. Jorma Vahala for providing the O₃ exposures during the mutant screening, Professor Sheng Yang He for providing the Pseudomonas strain used, and Professor Tapio Palva, Dr. Nigel Kilby, and Dr. Jörg Durner for their comments on the manuscript. This work was supported by Academy of Finland Grant Nos. 43671 and 37995 to J.K. and postdoctoral fellowship No. 41615 to H.T., by the Finnish Centre of Excellence Programme (2000-2005), and by grants from EU-FAIR (Brussels, Belgium) and Bundesministerium für Bildung und Forschung (BMBF) and Deutsche Forschungsgemeinschaft (DFG) (Bonn, Germany).

Received May 30, 2000; accepted July 10, 2000.

**REFERENCES**


Ozone-Sensitive Arabidopsis rcd1 Mutant Reveals Opposite Roles for Ethylene and Jasmonate Signaling Pathways in Regulating Superoxide-Dependent Cell Death
Kirk Overmyer, Hannele Tuominen, Reetta Kettunen, Christian Betz, Christian Langebartels, Heinrich Sandermann, Jr. and Jaakko Kangasjärvi

Plant Cell 2000;12;1849-1862
DOI 10.1105/tpc.12.10.1849

This information is current as of September 7, 2017

References
This article cites 43 articles, 16 of which can be accessed free at:
/content/12/10/1849.full.html#ref-list-1

Permissions

eTOCs
Sign up for eTOCs at:
http://www.plantcell.org/cgi/alerts/ctmain

CiteTrack Alerts
Sign up for CiteTrack Alerts at:
http://www.plantcell.org/cgi/alerts/ctmain

Subscription Information
Subscription Information for The Plant Cell and Plant Physiology is available at:
http://www.aspb.org/publications/subscriptions.cfm

© American Society of Plant Biologists
ADVANCING THE SCIENCE OF PLANT BIOLOGY