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Ethylene Synthesis Regulated by Biphasic Induction of 1-Aminocyclopropane-1-Carboxylic Acid Synthase and 1-Aminocyclopropane-1-Carboxylic Acid Oxidase Genes Is Required for Hydrogen Peroxide Accumulation and Cell Death in Ozone-Exposed Tomato[1]

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We show that above a certain threshold concentration, ozone leads to leaf injury in tomato (*Lycopersicon esculentum*). Ozone-induced leaf damage was preceded by a rapid increase in 1-aminocyclopropane-1-carboxylic acid (ACC) synthase activity, ACC content, and ethylene emission. Changes in mRNA levels of specific ACC synthase, ACC oxidase, and ethylene receptor genes occurred within 1 to 5 h. Expression of the genes encoding components of ethylene biosynthesis and perception, and biochemistry of ethylene synthesis suggested that ozone-induced ethylene synthesis in tomato is under biphasic control. In transgenic plants containing an *LE-ACO1* promoter-β-glucuronidase fusion construct, β-glucuronidase activity increased rapidly at the beginning of the O_3 exposure and had a spatial distribution resembling the pattern of extracellular H_2O_2 production at 7 h, which coincided with the cell death pattern after 24 h. Ethylene synthesis and perception were required for active H_2O_2 production and cell death resulting in visible tissue damage. The results demonstrate a selective ozone response of ethylene biosynthetic genes and suggest a role for ethylene, in combination with the burst of H_2O_2 production, in regulating the spread of cell death.

The gaseous plant hormone ethylene regulates many processes during plant growth and development and is also an important mediator of plant responses to biotic and abiotic stresses (Kende, 1993; Wang et al., 2002). The first committed step in ethylene biosynthesis, the conversion of *S*-adenosyl Met (Ado-Met) to 1-aminocyclopropane-1-carboxylic acid (ACC) is catalyzed by ACC synthase (ACS). ACC oxidase (ACO), in turn, oxidizes ACC to ethylene. ACC can also be conjugated to biologically inactive forms. In tomato (*Lycopersicon esculentum*), ACS and ACO are encoded by gene families consisting of at least eight (Oetiker et al., 1997; Shiu et al., 1998) and four members (Barry et al., 1996; Nakatsuka et al., 1998), respectively. These genes show differential expression during plant growth and development, and respond differentially to various external stimuli (Rottmann et al., 1991; Lincoln et al., 1993; Barry et al., 1996, 2000; Oetiker et al., 1997; Nakatsuka et al., 1998; Tatsuki and Mori, 1999; Llop-Tous et al., 2000).

Ozone (O_3) is a potent abiotic stress that induces ethylene synthesis in plants (Tingey et al., 1976; Kangasjärvi et al., 1994; Sandermann, 1996; Sandermann et al., 1998). Induction of ethylene synthesis by high

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O_3 is rapid, and a mechanistic connection between ethylene and O_3 damage has been demonstrated; when ethylene synthesis is prevented with ACS, ACO, or ethylene action inhibitors, or mutations in ethylene signaling in ozone-sensitive plants, tissue damage has been reduced accordingly (Mehlhorn and Wellburn, 1987; Bae et al., 1996; Tuomainen et al., 1997; Overmyer et al., 2000).

Ozone appears to act primarily as an elicitor of defense and damage-related processes and not directly as an oxidizing agent that damages leaf tissue (Schraudner et al., 1997; Sandermann et al., 1998; Overmyer et al., 2000; Rao and Davis, 2001; Langebartels et al., 2002). This seems to relate to the signaling function of oxygen radicals, and can be regarded as analogous to the oxidative burst in development of systemic acquired resistance and pathogen defense gene induction. The oxidative burst, corresponding to the release of reactive oxygen species (ROS) into the apoplastic space, is one of the earliest plant responses to pathogen infection (Lamb and Dixon, 1997; Dat et al., 2000). These ROS are also important components in regulating cell death in the hypersensitive response (HR), a form of programmed cell death (pcd) in plants (Levine et al., 1994; Tenhaken et al., 1995; Jabs et al., 1996; Alvarez et al., 1998).

In addition to the direct ROS formation from degradation of ozone in the apoplast, O_3 also induces an oxidative burst by the plant cells. In ozone-sensitive tobacco (*Nicotiana tabacum*) Bel W3 (Schraudner et al., 1998), birch (*Betula pendula*; Pellinen et al., 1999, 2002), ozone-sensitive Arabidopsis (Rao and Davis, 1999; Overmyer et al., 2000; Wohlgemuth et al., 2002), and native plant species (Wohlgemuth et al., 2002), H_2O_2 (tobacco and birch) and superoxide (Arabidopsis, *Malva sylvestris*, and *Rumex* sp.) production was evident in the tissues several hours after a short ozone pulse. The ROS production in Arabidopsis, tomato, and birch was partly inhibited by the plasma membrane NADPH oxidase inhibitor diphenylene iodonium accompanied with reduced tissue damage (Pellinen et al., 1999; Rao and Davis, 1999; Overmyer et al., 2000; Wohlgemuth et al., 2002).

The similarity between ozone- and pathogen-induced plant responses suggests that they may also be mechanistically similar. Ozone responses and damage appear to be a result of deleterious triggering of pcd associated with the HR, and ethylene seems to be centrally involved in the regulation of the processes (Rao and Davis, 2001; Langebartels et al., 2002). Evidence for a regulatory role for ethylene in pcd has also been obtained during pea (*Pisum sativum*) carpel senescence (Orzáez and Granell, 1997), in hypoxia-induced aerenchyma formation in maize (*Zea mays*) root cortex (He et al., 1996), in tomato cell cultures (de Jong et al., 2002), and in maize endosperm development (Young et al., 1997).

Our previous results showed that ozone exposure rapidly increased *LE-ACS2* and *LE-ACO* transcript levels (Tuomainen et al., 1997). *LE-ACO* mRNA levels were elevated already 30 min after the beginning of the stress and peaked at 1 h. However, ACO transcript levels, ACS activity, and ACC concentrations increased prior to the increase in *LE-ACS2* transcript levels, suggesting that posttranscriptional regulation could be involved in the increase of ACS activity in O_3-exposed tomato, or that other ACS gene family members may be induced prior to *LE-ACS2*. In this paper, we provide evidence that a second ACS gene, *LE-ACS6*, is rapidly induced by ozone prior to *LE-ACS2* and that individual members of the ACO and ethylene receptor gene families are differentially regulated following ozone. The temporal pattern of gene expression suggests that *ACS* and *ACO* gene expression is induced in a biphasic fashion in response to ozone. In addition, we show that there is a close correlation in the spatial location of the ethylene synthesis, ROS accumulation, and tissue damage, and that ethylene is required for ROS accumulation and subsequent cell death in tomato.

RESULTS

Ethylene Synthesis and Cell Death in Ozone-Exposed Tomato

Ozone-exposed plants synthesize significant amounts of ethylene at the beginning of the exposure when a threshold level of O_3, which varies between different species and cultivars, is exceeded. The O_3 threshold and ethylene evolution for the tomato cv Ailsa Craig was determined by exposing plants to 0 to 250 nL L^{-1} O_3 for up to 5 h, followed by clean air. Ethylene evolution increased in a dose-dependent manner in middle-aged leaves (three and four from the apex; Fig. 1A) and was accompanied by increased ACS activity and ACC accumulation (data not shown). In the control plants and plants exposed to 100 nL L^{-1} O_3, no significant changes were observed in ethylene evolution, but in plants exposed to higher than 150 nL L^{-1}, pronounced increases in ethylene evolution (Fig. 1A) were clearly visible 1.5 h after the initiation of the exposure. O_3 concentrations of 200 and 250 nL L^{-1} caused slightly higher ethylene production. Tissue damage in leaves of different ages, visible 24 h after the exposure, was dependent on the O_3 concentration (Fig. 1B). At 100 nL L^{-1}, no damage was evident in any leaves, whereas at 200 nL L^{-1}, the overall damage was significantly higher and the extent of damage increased from leaf three to six. When 250 nL L^{-1} O_3 was used, most of the damage was on leaves three to five, which also showed the highest ethylene evolution (data not shown).

The Expression of Specific *LE-ACS*, *LE-ACO*, and Ethylene Receptor Gene Family Members Is Differentially Regulated by Ozone

Our previous results (Tuomainen et al., 1997) showed that of the four ACS genes studied, O_3 in-

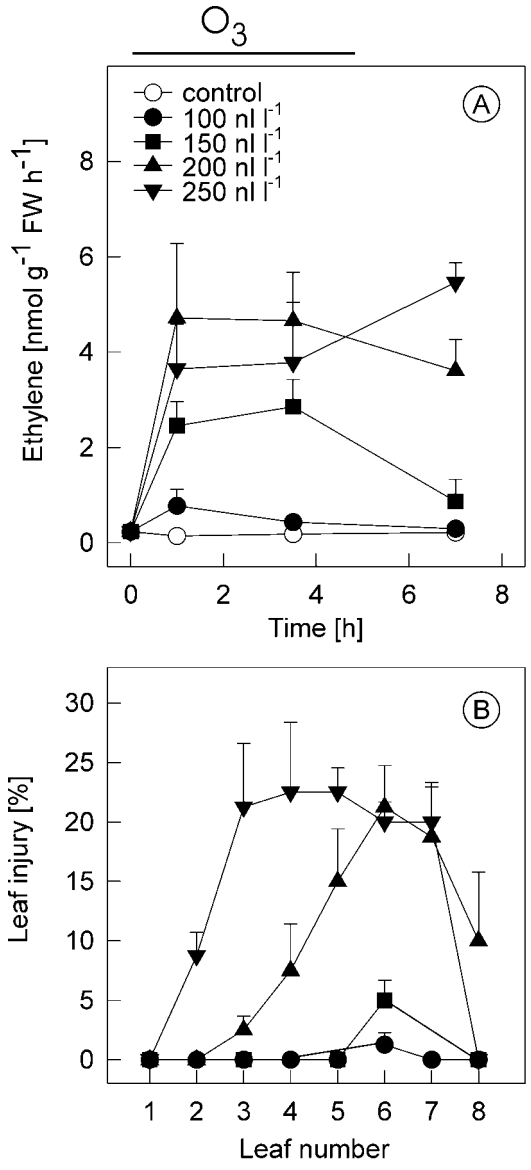

Figure 1. Ozone induction of ethylene emission and tissue damage in transgenic cv Ailsa Craig (AC) tomato harboring an *LE-ACO1*::*uidA* construct. A, Dose response of ethylene evolution. cv Ailsa Craig plants were exposed to 0 to 250 nL L^{-1} ozone for 5 h (indicated by the line above the graph). Ethylene evolution was determined from plants collected at times shown. B, Leaf injury in ozone-exposed *LE-ACO1*::uidA plants treated as in A. Leaf injury was assessed 24 h later in leaves 1 to 8 from the top of the plant. Means ± SE (n = 3).

creased only *LE-ACS2* transcript levels. In addition, using a generic ACC oxidase probe, pTOM13 that hybridizes to all of the ACO gene family members, we found that ACO transcript abundance increased more rapidly than *LE-ACS2*. Based on the published sequence (Oetiker et al., 1997), we cloned a fragment of *LE-ACS6* and used it with the *LE-ACS1A*, *LE-ACS1B*, and *LE-ACS2* gene-specific probes (Barry et al., 2000) in ribonuclease protection assay (RPA). *LE-ACS1A* expression was extremely low in leaves, transcript abundance was not altered by O_3, and *LE-ACS1B* transcripts were below the limits of detection (data not shown). However, *LE-ACS6* was up-regulated within 1 h of the beginning of the O_3 treatment, declined at 2 h to the level before the treatment, and was below the initial level through 5 h. In contrast, *LE-ACS2* was not induced until 2 h after the beginning of the exposure and increased through 5 h (Fig. 2).

All four ACO gene family members were expressed in O_3-treated leaves, although the expression pattern of individual members differed (Fig. 2). *LE-ACO1* and *LE-ACO3* transcripts increased within 1 h of the beginning of the treatment. However, whereas *LE-ACO1* transcripts remained elevated throughout the duration of the experiment, *LE-ACO3* transcripts showed only a transient increase, with diminishing levels by 5 h. *LE-ACO4* transcripts remained unchanged until 5 h when a slight increase was observed. *LE-ACO2* transcripts also showed a slight induction 5 h after the beginning of the treatment.

Expression of the ethylene receptor genes *LE-ETR1*, *LE-ETR2*, and *NR* (*LE-ETR3*) were similarly analyzed

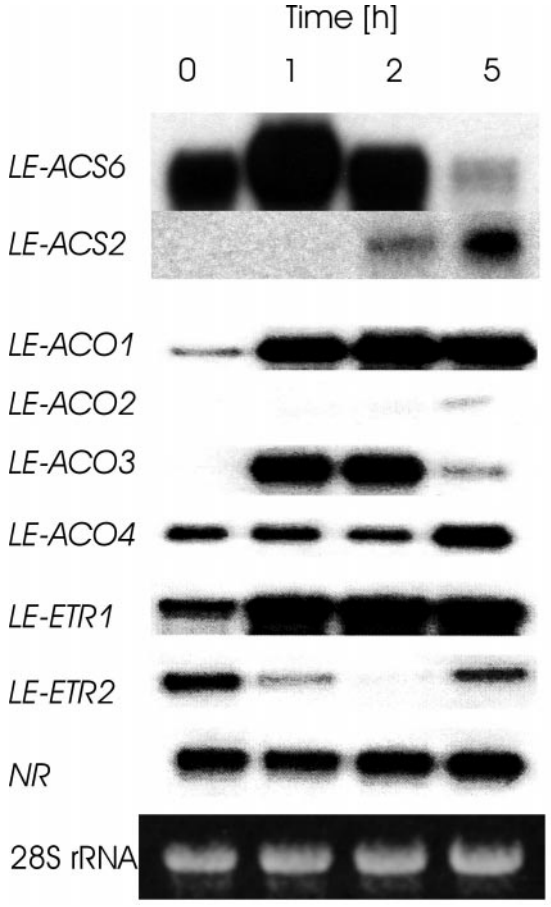

Figure 2. ACC synthase, ACC oxidase, and ethylene receptor gene expression in ozone-exposed leaves. Twenty micrograms of total RNA extracted from ozone-exposed (200 nL L^{-1} for 0–5 h) leaves of cv Ailsa Craig were hybridized with radiolabeled gene-specific probes, and RPA analysis was performed. Equal amount of RNA used in the RPAs is shown below the autoradiographs.

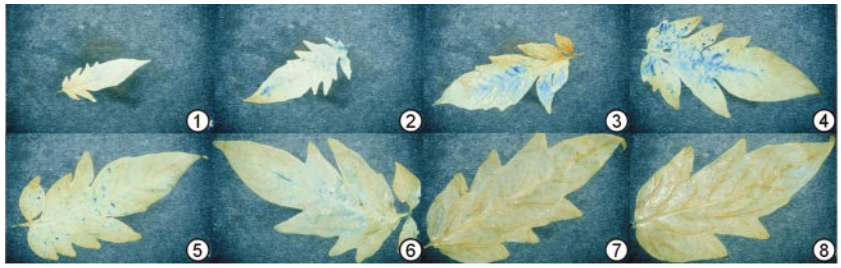

Figure 3. Histochemical localization of ACC oxidase activation in the leaves of ozone-exposed transgenic *LE-ACO1* promoter::*uidA* plants. The effect of ozone (250 nL L^{-1} for 30 min) on GUS activity in leaves of various ages. The terminal leaflets were collected from leaves 1 through 8 counting from the top of the plant.

by RPA. O_3 caused the transcript levels of *LE-ETR1* to increase 1 h after the beginning of the exposure. Expression of NR was unaffected by O_3, whereas the transcripts of *LE-ETR2* decreased markedly 1 h after the beginning of the exposure, and by 2 h, the transcripts had disappeared below the detection level. However, 5 h after the beginning of the exposure, the transcripts of *LE-ETR2* returned again to almost the same levels as at the beginning of the exposure.

Spatial Localization of *LE-ACO1* Activation by Ozone in Transgenic *ACO1* Promoter-β-Glucuronidase (GUS) Fusion Plants

To give an indication of the spatial location of ethylene synthesis in response to O_3, we examined *LE-ACO1* promoter-driven GUS activity in leaves of different ages in response to 250 nL L^{-1} O_3. In terminal leaflets collected from leaves 1 to 8 during the exposure, the most prominent GUS staining was in leaves 3 and 4 (Fig. 3), which were also sensitive to O_3 and showed tissue damage in these experiments (data not shown). GUS activity was visible as spots located in the interveinal tissue in close vicinity to the veins. No staining covering large, continuous leaf areas was detected. In the youngest leaves, O_3 did not cause visible GUS staining in the terminal leaflet (Fig. 3) or in other leaflets (not shown). In leaves six and older, GUS activity was not detected. The oldest leaves used in these experiments with *LE-ACO1::GUS* plants were still fully green, had not yet started to senesce, and did not show O_3 damage (data not shown).

Localization of Ethylene Synthesis, H_2O_2 Production, and Tissue Damage

We determined histochemically the accumulation of $O_2^{-\bullet}$ and H_2O_2 in the O_3-exposed plants and compared it with the location of ACC oxidase gene activation by O_3. Similar to our results (Wohlgemuth et al., 2002) in various commercial tomato cultivars, superoxide-specific nitroblue tetrazolium (NBT) staining did not reveal any detectable NBT precipitation in the tomato leaves (data not shown). The positive control, an O_3-sensitive Arabidopsis mutant that produces $O_2^{-\bullet}$ after O_3 (Overmyer et al., 2000), displayed clear $O_2^{-\bullet}$-dependent NBT staining under identical conditions. To study the relationship between the H_2O_2 accumulation and the following damage formation, one-half of a leaflet was collected for the determination of H_2O_2 accumulation, and the other one-half of the same leaflet was used 24 to 48 h later to determine the spatial location of cell death. Histological staining for H_2O_2 by 3,3′-diaminobenzidine 4 HCl (DAB; Thordal-Christensen et al., 1997) showed local accumulation of H_2O_2 in the vicinity of the veins at 7 h (Fig. 4B), corresponding closely to the damage pattern that was visible in the other leaflet one-half 24 to 48 h later (Fig. 4C). The GUS staining pattern at 2 h had a similar spatial pattern (Fig. 4A) as those regions showing H_2O_2 accumulation at 6 h (Fig. 4B), and cell death 24 to 48 h later (Fig. 4C).

To determine the cellular location of the ACO1-promoter-driven GUS activity, cross-sections were cut through the GUS-positive sites of the O_3-exposed leaves. The distribution of GUS staining was detectable through spongy and palisade parenchyma, but

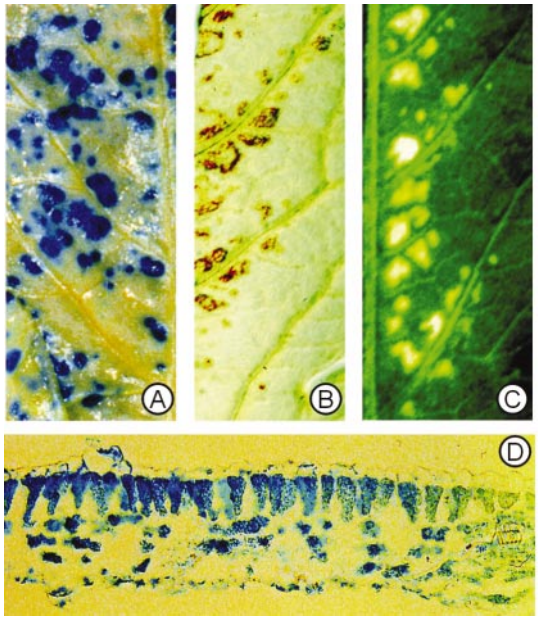

Figure 4. Cellular and tissue localization of ACC oxidase activation, H_2O_2 accumulation, and visible injury. Plants were exposed to 250 nL L^{-1} ozone for 5 h, and terminal leaflets from leaf number 2 from ozone-treated plants were analyzed for GUS activity regulated by the *LE-ACO1* promoter 1 h after the beginning of the exposure (A), H_2O_2 accumulation 7 h after the beginning of the exposure (B), and ozone symptom localization after 24 h (C). D, Transverse section through a leaflet showing *LE-ACO1* expression indicated by GUS staining at 1 h.

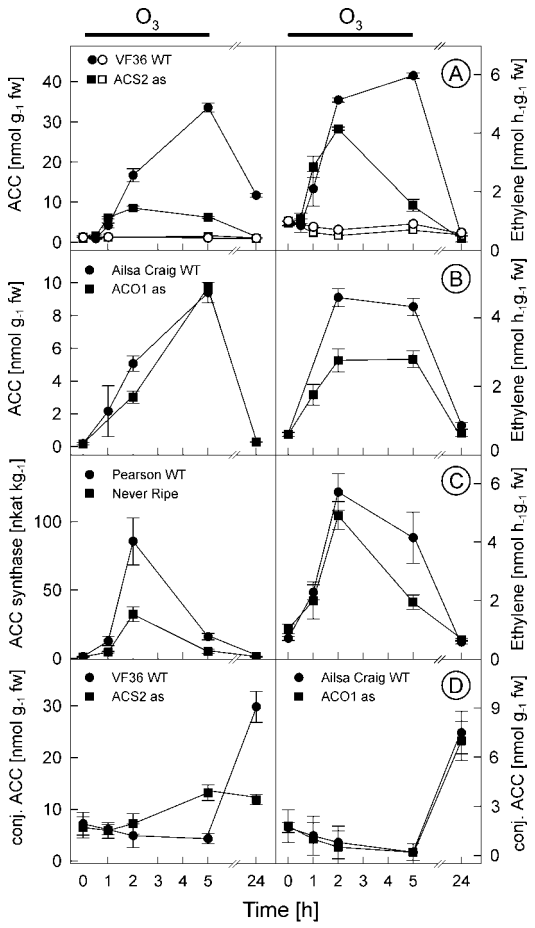

Figure 5. Ethylene synthesis in ozone-exposed tomatoes deficient in ethylene biosynthesis or perception. ACC concentrations and ethylene evolution were measured from ozone-exposed (filled symbols) and clean air control (blank) tomato wild-type cv VF-36 and transgenic *LE-ACS2* antisense plants (A) and from ozone-exposed cv Ailsa Craig and transgenic *LE-ACO1* antisense plants (B). C, ACC synthase activity and ethylene evolution were measured from ozone-exposed cv Pearson and ethylene-insensitive *Nr* mutant. D, Concentrations of conjugated ACC were measured from ozone-exposed VF36, *LE-ACS2* antisense (in cv VF36), cv Ailsa Craig, and *LE-ACO1* antisense (in cv Ailsa Craig) tomatoes during and after the 5-h ozone exposure. Plants were treated for 5 h with 200 nL L^{-1} ozone (indicated with black line above the graphs) and were postcultivated in pollutant-free air. Means ± SE ($n = 3$).

was not present in the epidermal cells (Fig. 4D). However, in the more distal regions of the spots, GUS activity was confined more to the palisade cells.

Ethylene Biosynthesis and Perception Are Required for the H_2O_2 Synthesis and Cell Death in Tomato

Because there was a very close correlation between the location of ethylene and H_2O_2 synthesis (Fig. 4) and subsequent cell death, we examined whether ethylene synthesis and perception are required for the production of H_2O_2 in tomato. Transgenic tomato plants harboring the *LE-ACS2* gene in antisense orientation (Oeller et al., 1991) accumulated ACC and emitted ethylene during the first 2 h of O_3 exposure to a similar degree as the untransformed control plants (cv VF36); however, after 2 h, ACC concentration and ethylene evolution in the antisense plants did not increase as in the wild-type plants (Fig. 5A). As a consequence, there was no difference in the O_3 sensitivity between the wild-type and *LE-ACS2* antisense plants (data not shown). ACC accumulation and the subsequent ethylene evolution was the result of de novo ACC synthesis because the conjugated ACC levels did not change during the exposure to such degree that release of ACC from the conjugated forms could account for the increase in ACC (Fig. 5D). However, 24 h after the exposure, the concentrations of conjugated ACC increased in cv Ailsa Craig, *LE-ACO1* antisense plants, and in the

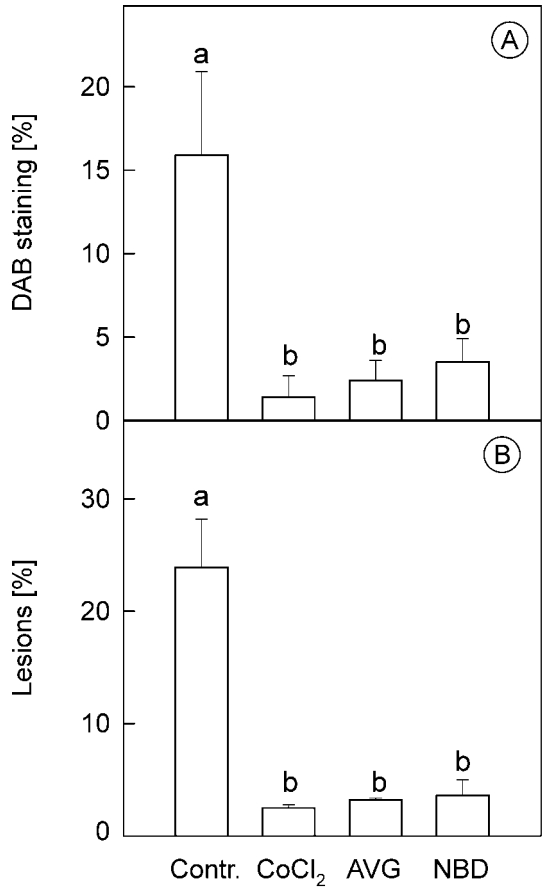

Figure 6. The effect of inhibition of ethylene biosynthesis or perception on lesion development and H_2O_2 accumulation. Plants were treated before ozone exposure with the ACC synthase inhibitor AVG, ACC oxidase inhibitor $CoCl_2$, or the ethylene antagonist NBD. Inhibitor-treated plants or untreated controls were then exposed to 250 nL L^{-1} ozone for 5 h. A, One-half of the leaves were harvested 7 h after the beginning of the exposure and were stained for H_2O_2 production with DAB. B, Cell death (indicated as the percentage lesion of the leaf area) measured after 24 h. Means ± SE ($n = 3$). Columns with the same letter are not significantly ($P < 0.05$) different according to Tukey's multiple range test.

VF36 wild type, but not in the *LE-ACS2* antisense plants (Fig. 5D) that had also low ACC concentrations (Fig. 5A).

In a similar manner, transgenic tomato plants harboring the *LE-ACO1* in antisense orientation (Hamilton et al., 1990) were exposed to O_3. Even though these plants show highly reduced ethylene evolution in the developing fruit, in the leaves of the antisense plants, ethylene evolution was reduced by only about 50% (Fig. 5B), and the plants did not differ from the wild-type cv Ailsa Craig in their O_3 sensitivity. The remaining ethylene evolution in the LE-ACO1 antisense plants was obviously over the threshold limit (Tuomainen et al., 1997) that is sufficient to stimulate lesion formation. In the *LE-ACO1* antisense plants, the concentration of free ACC did not differ from the wild-type cv Ailsa Craig during the course of the experiment (Fig. 5B).

Leaves of the *Never-ripe* (*Nr*) mutant, which carries a dominant mutation in the ethylene receptor *LE-ETR3* (Wilkinson et al., 1995), did not differ from the corresponding wild-type cv Pearson in O_3 sensitivity. However, there was a clear difference in ACC synthase activity between cv Pearson and *Nr* in response to O_3 (Fig. 5C). During the 2nd h of the exposure, ACS activity was so high in the cv Pearson control that Ado-Met concentration was most likely the limiting factor for ethylene synthesis because in *Nr*, ethylene evolution was similar as in the wild type.

To completely block the enzymes of ethylene biosynthesis or perception in the leaves, plants were treated with the ethylene biosynthesis inhibitors aminoethoxyvinyl Gly (AVG) and Co^{2+}, and the ethylene perception antagonist norbornadiene (NBD). Inhibition of ACS activity with AVG, ACO activity with Co^{2+}, or ethylene perception with NBD all reduced H_2O_2 accumulation at 6 to 8 h significantly in O_3-exposed plants (Fig. 6A), and tissue damage at 24 h was decreased accordingly (Fig. 6B). This demonstrated that ethylene synthesis and perception are required for the oxidative burst and for the progression of O_3-induced lesions in the leaves to take place.

DISCUSSION

Ozone Differentially Induces Genes Involved in Ethylene Synthesis and Perception

Previous studies on tomato have shown that individual members of the ACS, ACO, and ETR gene families are differentially expressed during developmental processes and in response to external stimuli (Rottmann et al., 1991; Lincoln et al., 1993; Barry et al., 1996, 2000; Oetiker et al., 1997; Lashbrook et al., 1998; Nakatsuka et al., 1998; Tatsuki and Mori, 1999; Tieman and Klee, 1999; Ciardi et al., 2000; Llop-Tous et al., 2000). We have examined the expression of these gene families in response to a single 5-h pulse of the air pollutant O_3. The changes in expression patterns seen in response to O_3 can be grouped into two classes based on temporal changes in expression. Rapid changes, within 1 h of the beginning of the treatment, were seen for *LE-ACS6*, *LE-ACO1*, *LE-ACO3*, *LE-ETR1*, and *LE-ETR2*, and slow changes, occurring after 2 h, were seen for *LE-ACS2*, *LE-ACO2*, and *LE-ACO4* (Fig. 2).

These data suggest a biphasic regulation of these genes in response to O_3, and this observation is supported by measurements of ACC content and ethylene production in *LE-ACS2* antisense plants (Fig. 5). ACC content and ethylene production increased rapidly in the VF36 control and *LE-ACS2* antisense plants up to the first 1 to 2 h after the beginning of the treatment, after which there was a rapid decline (Fig. 5A). Based upon our expression data, it is likely that this initial increase (phase 1) was due to the rapid induction of *LE-ACS6* (Fig. 2). However, 2 h after the beginning of the exposure, when *LE-ACS2* was induced (Fig. 2) and the antisense effect is activated, sustained ACC accumulation and ethylene synthesis was prevented in the *LE-ACS2* antisense plants. A similar pattern is seen in ACS activity in cv Pearson control and *Nr* mutant plants (Fig. 5C), which suggests that at around 2 h after the beginning of the O_3 treatment, the increase observed in ACS activity in control plants is ethylene receptor-dependent, suggesting deficient negative feedback regulation of ACS activity in the O_3-exposed *Nr* plants.

A biphasic relationship between *LE-ACS2* and *LE-ACS6* expression has previously been shown in response to wounding of tomato leaves and mature green fruit (Tatsuki and Mori, 1999). The rapid and transient induction of *LE-ACS6* expression in response to wounding was followed by the later accumulation of *LE-ACS2* transcripts. *LE-ACS6* is regulated by negative feedback (Nakatsuka et al., 1998), which can also be deduced by comparing Figures 1 and 2, which show down-regulation of *LE-ACS6* at the same time when *LE-ACS2* is activated and ethylene evolution continues to increase. Together, these data suggest that a biphasic mode of *ACS* and possibly also *ACO* transcript accumulation may be a feature of stress-induced ethylene synthesis in tomato. However, it is not yet understood how the signal from these diverse input stimuli results in a common response mechanism, and more stress responses need to be investigated to see if this relationship holds true.

It is possible that biphasic control of ethylene synthesis may have evolved as a regulatory mechanism to modulate plant responses depending upon the severity of the stress encountered. For example, O_3 and mechanical wounding appear to result in a biphasic induction of *ACS* gene expression (Fig. 2; Tatsuki and Mori, 1999). In contrast, the comparatively milder stress of touch results in the rapid induction of *LE-ACS1A* and *LE-ACS6*, but does not lead to a later higher induction of *LE-ACS2* (Tatsuki and Mori, 1999). Of course, it is also possible that the differen-

tial expression of ACS and ACO genes in response to ozone treatment may occur at the cellular level and may be directly related to cell damage. The results in Figure 4 indicate that *LE-ACO1* expression shows spatial specificity in response to ozone treatment. It will be of interest to determine whether cell-specific expression is shown by other members of the ACS and ACO gene families in response to ozone treatment and other stresses.

Ethylene Is Involved in the Regulation of the Degree of Ozone Damage

Ethylene emission has been shown to correlate to ozone sensitivity in several plant species. It was initially proposed that O_3 could react chemically with ethylene and form radicals that in turn would damage the biological structures of the cells (Elstner et al., 1985; Mehlhorn and Wellburn, 1987). However, more recent results suggest that ethylene plays a more active role in O_3 damage. Our previous results indicated reduced O_3 damage when ethylene synthesis was prevented with inhibitors of ACC synthase or oxidase (Tuomainen et al., 1997). In a similar manner, use of the ethylene antagonist NBD reduced O_3-induced lesion formation in tomato (Bae et al., 1996), and in Arabidopsis mutants selected for increased sensitivity to O_3 (Overmyer et al., 2000), ethylene evolution was triggered during the early lesion development. Thus, existing sensitivity of the genotype to O_3 (tomato and tobacco) and gain of sensitivity by mutation in resistant background (Arabidopsis; Overmyer et al., 2000) involve rapid activation of ethylene biosynthesis in the tissues that show subsequent hallmarks of pcd.

Ethylene is involved in pcd during developmental and inducible processes (He et al., 1996; Orzáez and Granell, 1997; Young et al., 1997; de Jong et al., 2002). Our results suggest that ethylene has also an intimate role in the regulation of early O_3 lesion development. Disease lesion development also requires ethylene action in tomato and Arabidopsis (Bent et al., 1992; Lund et al., 1998). Together, these results suggest an active role for ethylene in regulating the spread of lesions, though the exact mechanisms of disease lesion development and the possible involvement of ROS and pcd therein are not known.

The role of ROS and Ethylene in Cell Death Signaling

Pcd is involved in several developmental and inducible processes in plants. In the HR to incompatible pathogens, which is one of the best-studied forms of pcd in plants (Dangl et al., 1996; Levine et al., 1996; Pennell and Lamb, 1997), two separate ROS bursts take place. In a similar manner, in O_3-sensitive tobacco cv Bel W3, two separate O_3-induced bursts were detected (Schraudner et al., 1998). The second burst was correlated in distribution and size with the lesions that appeared later, and was absent in the O_3-tolerant cv Bel B. We have begun to address the spatial location of H_2O_2 accumulation and ethylene biosynthesis in response to O_3 with the aid of transgenic plants expressing an *LE-ACO1* promoter::GUS fusion (Figs. 3 and 4). The results indicated that rather than expression throughout the leaf, H_2O_2 accumulation and GUS expression were confined to distinct regions surrounding the vascular tissue, mainly in the parenchyma cells. This restricted expression is of interest as clearly not all cells are responding to O_3 in the same way.

In O_3-exposed plants, ROS formation from the degradation of O_3 is not confined to a limited location as in the HR; O_3 enters the substomatal cavities all over the leaf. However, ethylene synthesis, H_2O_2 accumulation, and the subsequent lesion development took place in clusters of cells close to the vasculature (Fig. 4). This colocalization may favor interaction of these signal molecules as it predicts that high concentrations co-occur in the same cells. In addition, the spatial location close to the veins is similar to the location of ROS generation during the HR, which is essential in the establishment of systemic resistance (Alvarez et al., 1998). In a similar manner, cell death that is preferentially localized to cells close to the vascular bundles was seen in O_3-exposed tobacco (Schraudner et al., 1998), tomato, *M. sylvestris*, and Arabidopsis (Wohlgemuth et al., 2002). As discussed by Schraudner et al. (1998), the cells in the periveinal region might be disposed to amplify ROS production ("burst initiation sites"; Schraudner et al., 1998) and to die during the pathogenesis response, i.e. to limit pathogen spread via the vascular system.

Our results suggest an integral role for ethylene in the regulation of cell death. Ethylene seems to be involved in the regulation of cell death by amplifying a second burst of ROS production. When ethylene synthesis or perception was prevented with inhibitors, the second oxidative burst was inhibited, and accordingly, tissue damage was also reduced. In agreement with the model of the oxidative cell death cycle, originally proposed by van Camp et al. (1998) and modified by Overmyer et al. (2000), these results indicate that ethylene is intimately involved in the amplification of ROS production and regulation of cell death under oxidative stress.

MATERIALS AND METHODS

Plant Material and Conditions of Treatment

Tomato (*Lycopersicon esculentum* cv Ailsa Craig, cv VF36, and cv Pearson) plants were grown in pollutant-free air under a 14-h/10-h light/dark regime (at 100 μmol m^{-2} s^{-1} from 6 AM to 8 PM) at 25°C/20°C as described previously (Tuomainen et al., 1997). Six- to 7-week-old plants were exposed to a single pulse of O_3 (<5–300 ± 10 nL L^{-1}) for 0.5 to 7 h (starting at 9 AM). Ozone concentrations were 0, 100, 150, 200, 250, and 300 nL L^{-1} in dose-response experiments, and 0 and 200 nL L^{-1} in time course experiments. Ozone was generated by electric discharge in dry oxygen and was measured with an analyzer (CSI 3100; Messer-Griesheim, Munich, Germany), periodically calibrated as described (Langebartels et al., 1991). Control plants were cultivated in pollutant-free air in parallel chambers.

Inhibitors of ethylene biosynthesis (AVG; Sigma, St. Louis, and cobalt chloride) and perception (NBD; Sigma) were applied as described (Bae et al., 1996; Tuomainen et al., 1997).

Leaves were numbered from the apex of the plants with leaf number 2 larger than 13 cm. Analyses were routinely performed with the O_3-sensitive middle-aged leaves numbers 3 to 5. Injury was scored 24 h after the onset of exposure by assessing visible leaf injury as percentage of leaf area. The data were then calibrated with a planimeter (LI3000A, LI-COR, Lincoln, Nebraska). Gas exchange measurements were performed with a portable porometer (CQP 130a; Walz, Effeltrich, Germany) according to Langebartels et al. (1991). For biochemical analyses, leaves were immediately frozen in liquid nitrogen and were stored at −75°C.

Determination of Ethylene Production in Situ

Individual leaflets from leaves numbers 3 to 5 (approximately 0.2 g of fresh weight with the cut surface sealed with liquid paraffin) were placed adaxially on water-moistened filter papers. The papers were rolled cylindrically and placed into glass tubes, which were then sealed by silicone septa. After incubation at room temperature for 1 h in the dark, 1-mL gas samples were withdrawn with a syringe, and ethylene was analyzed according to Tuomainen et al. (1997).

Determination of ACC Contents

Leaf material was ground in liquid nitrogen and was extracted according to Langebartels et al. (1991). ACC and total ACC following acid hydrolysis (2 N HCl for 3 h at 120°C) were determined according to Lizada and Yang (1979) as described (Langebartels et al., 1991). The amount of conjugated ACC was calculated by subtracting the amount of ACC from that of total ACC.

Determination of ACC Synthase Activity

Frozen leaves (0.2 g) were ground in liquid N_2 and were extracted with 0.5 mL of 100 mM EPPS [4–2(2-hydroxyethyl)-1-piperazine propane sulfonic acid] buffer, pH 8.5, containing 5 mM DL-dithiothreitol, 5 μM pyridoxal phosphate, and protease inhibitors (500 μM phenylmethylsulfonyl fluoride and 10 μM leupeptin). After the addition of water-insoluble polyvinylpolypyrrolidone (2%, w/v) and vortexing for 10 s, the extract was centrifuged at 20,000*g* for 10 min at 4°C. The supernatant was gel-filtered on a Sephadex G-25 column (NAP-5 column; Pharmacia, Freiburg, Germany) equilibrated with 5 mM EPPS (pH 8.5), 1 mM DL-dithiothreitol, 5 μM pyridoxal phosphate, and 500 μM phenylmethylsulfonyl fluoride. ACC synthase was assayed in glass flasks containing 0.4 mL of protein extract and final concentrations of 80 mM EPPS (pH 8.5), 20 μM pyridoxal phosphate, and 100 μM Ado-Met, in a total volume of 0.5 mL at 30°C for 2 h (Tuomainen et al., 1997). Blanks omitting Ado-Met were incubated in parallel. The reaction was stopped by addition of 100 μL of 10 mM $HgCl_2$ on ice. ACC was converted to ethylene as described above. One milliliter of the gas phase was withdrawn by simultaneously adding 1 mL of water through a second syringe and was analyzed by gas chromatography.

Isolation of RNA, Nucleic Acid Probes, and RNase Protection Assay

Total RNA was extracted from frozen, homogenized leaf tissue as described by Chang et al. (1993). Poly(A)$^+$ RNA was extracted from 300 to 500 μg of total RNA using the PolyATtract mRNA Isolation System IV (Promega, Madison, WI). Gene-specific probes for *ACS* and *ACO* sequences were as described previously (Barry et al., 1996, 2000; Llop-Tous et al., 2000). Gene-specific probes for the ethylene receptor genes, *LE-ETR1*, *LE-ETR2* (Lashbrook et al., 1998), and *NR* (Wilkinson et al., 1995) were designed from around the 3′end of each sequence. Primer pairs were as follows: *LE-ETR1*, ETR1F: 5′-tagtgaatgtaggaggaaaa-3′ and ETR1R: 5′-cacataataatctattgttg-3′, generating a probe from nucleotides 2,308 to 2,621; *LE-ETR2*, ETR2F: 5′-cagtaaaccaaaattgtctc-3′ and ETR2R: 5′-gactgtcattgtatttttct-3′, generating a probe from nucleotides 2,324 to 2,589; and *NR*, NRF: 5′-taaatgacaaaaggacat-3′ and NRR: 5′-gtcaaaagctcgatgtat-3′, generating a probe from nucleotides 2,210 to 2,399. PCR products were cloned into the pCR2.1 vector (Invitrogen, San Diego). The RNase protection assay to analyze the gene-specific ACC oxidase transcript abundance was performed as described earlier (Barry et al., 1996, 2000).

GUS Activity Determination

GUS activity was localized histochemically by placing detached leaflets in the staining buffer containing 0.5 mM 5-bromo-4-chloro-3-indolyl-β-glucuronic acid (Calbiochem, La Jolla, CA) following procedure described (Blume and Grierson, 1997). After vacuum infiltration of the buffer, samples were incubated in darkness at 37°C overnight. For microscopical localization of GUS activity within the leaf, sections of stained leaves were fixed, embedded in paraffin, and transverse sections were cut through the GUS-positive spots.

H_2O_2 Detection by DAB Staining

Individual leaflets from middle-aged leaves were infiltrated with 0.1% (w/v) DAB, 10 mM MES (pH 6.5; Thordal-Christensen et al., 1997) or 0.1% (w/v) NBT, 10 mM sodium azide, and 50 mM potassium phosphate (pH 6.4; Jabs et al., 1996). Leaves were incubated in the light for 30 min and were then cleared in ethanol for 2 d at room temperature in the dark (Wohlgemuth et al., 2002).

Statistical Analysis

All experiments were conducted in a completely randomized design with three replicates for each treatment. When indicated, the Tukey multiple range test was used to test for differences among treatment means (at $P = 0.05$; Statgraphics software; STSC, Rockville, MD).

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