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Differential Effects of Elevated Ozone on Two Hybrid Aspen Genotypes Predisposed to Chronic Ozone Fumigation. Role of Ethylene and Salicylic Acid[1]

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The role of ethylene (ET) signaling in the responses of two hybrid aspen (*Populus tremula* L. × *P. tremuloides* Michx.) clones to chronic ozone (O_3; 75 nL L^{-1}) was investigated. The hormonal responses differed between the clones; the O_3-sensitive clone 51 had higher ET evolution than the tolerant clone 200 during the exposure, whereas the free salicylic acid concentration in clone 200 was higher than in clone 51. The cellular redox status, measured as glutathione redox balance, did not differ between the clones suggesting that the O_3 lesions were not a result of deficient antioxidative capacity. The buildup of salicylic acid during chronic O_3 exposure might have prevented the up-regulation of ET biosynthesis in clone 200. Blocking of ET perception with 1-methylcyclopropene protected both clones from the decrease in net photosynthesis during chronic exposure to O_3. After a pretreatment with low O_3 for 9 d, an acute 1.5-fold O_3 elevation caused necrosis in the O_3-sensitive clone 51, which increased substantially when ET perception was blocked. The results suggest that in hybrid aspen, ET signaling had a dual role depending on the severity of the stress. ET accelerated leaf senescence under low O_3, but under acute O_3 elevation, ET signaling seemed to be required for protection from necrotic cell death.

The gaseous phytohormone ethylene (ET) is a signal molecule that is active during both plant development and senescence and is synthesized as a response to several biotic and abiotic stresses (Tingey et al., 1976; Yang and Hoffman, 1984; Abeles et al., 1992; Johnson and Ecker, 1998). The ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC) is synthesized from *S*-adenosyl-L-Met by ACC synthase (ACS; Yang and Hoffman, 1984). ACC is further converted to CO_2, cyanide (HCN), and ET by ACC oxidase. The HCN formed is rapidly detoxified by β-cyano-Ala synthase (β-CAS; Yip and Yang, 1988). ET has a role in the regulation of several plant defense genes (Broglie et al., 1986; Ecker and Davis, 1987; Eyal et al., 1993; Leubner-Metzger et al., 1998), but it also has an opposite role; ET can promote cell death under oxidative stress (Overmyer et al., 2000) and pathogen attack (Ciardi et al., 2001).

Tropospheric ozone (O_3) has been recognized as a severe air pollutant since the 1950s. Ambient O_3 concentrations in northern Europe vary usually between 20 to 50 nL L^{-1} during the growth season, but acute O_3 peaks exceeding 150 nL L^{-1} are also regularly observed. Once O_3 enters the leaf through stomata, it degrades rapidly in the apoplast to form various reactive oxygen species (ROS; Mehlhorn et al., 1990; Kanofsky and Sima, 1995; Langebartels et al., 2002). It has been proposed that this apoplastic ROS formation may alter the integrity of the plasma membrane and thus cause cellular damage (Laisk et al., 1989; Heath and Taylor, 1997). To remove and detoxify excess ROS, plants have both enzymatic and nonenzymatic antioxidant defenses such as ascorbic acid, glutathione, α-tocopherol, and catalase (Noctor and Foyer, 1998; Vanacker et al., 1998). Of these, glutathione is regarded as a central component for antioxidant defense in most aerobic organisms (Foyer et al., 1997). It is also more prone to oxidation than, for example, ascorbate (Polle, 2001).

Under various stress conditions, ROS can also serve as a signal that triggers defense-related molecular responses through the regulation of gene expression or enzyme activities (Lamb and Dixon, 1997). For example, in the incompatible plant-pathogen interaction, a self-propagating formation of apoplastic ROS is an essential signal that regulates cell death in the hypersensitive response (Lamb and Dixon, 1997).

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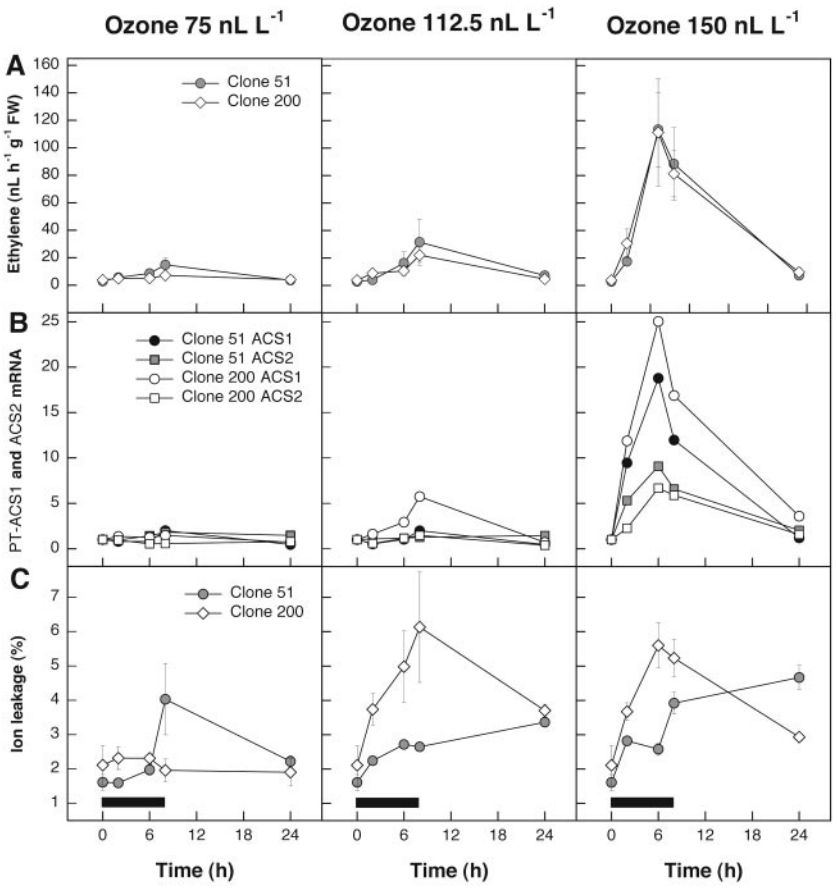

Figure 1. O_3-induced ET evolution (A), induction of the ACS genes *PT-ACS1* and *PT-ACS2* (B), and O_3-induced ion leakage (C) in hybrid aspen clones 51 and 200 in response to a single 8-h pulse of 75, 112.5, and 150 nL L^{-1} O_3. ET evolution and ion leakage were measured from the first and third fully expanded leaves. Error bars indicate ±SE ($n = 3$). Black bars indicate the duration of O_3 fumigation. Transcript levels are expressed as relative to hybridization signal at 0 h.

It has been shown that also O_3 induces active ROS production in planta and elicits programmed cell death (PCD) via the activation of the oxidative burst (Schraudner et al., 1998; Pellinen et al., 1999, 2002; Rao and Davis, 1999; Overmyer et al., 2000; Wohlgemuth et al., 2002). According to the current understanding, the ROS formed from O_3 and the subsequent oxidative burst in planta act more as signal molecules that elicit plant responses and induce PCD, rather than as directly oxidizing agents (Kangasjärvi et al., 1994; Rao et al., 2000; Langebartels et al., 2002).

The involvement of ET in determining the degree of O_3 lesion formation has been demonstrated in several plant species, and attenuation of ET biosynthesis has been shown to reduce O_3-induced cell death substantially (Mehlhorn and Wellburn, 1987; Mehlhorn et al., 1991; Schlagnhaufer et al., 1995; Wenzel et al., 1995; Tuomainen et al., 1997; Moeder et al., 2002). However, the role of functional ET perception and signaling in response to O_3 is less studied. ET perception can be prevented pharmacologically with, for example, norbornadiene (NBD) or 1-methylcyclopropene (MCP; Sisler and Serek, 1997). The use of NBD significantly reduced visible O_3 damage in tomato (*Lycopersicon esculentum*; Bae et al., 1996; Moeder et al., 2002). Similarly, in an O_3-sensitive Arabidopsis mutant *rcd1*, NBD prevented the propagation of O_3-induced superoxide accumulation and cell death, which suggested a crucial role for ET to promote cell death via PCD (Overmyer et al., 2000). O_3-induced salicylic acid (SA) accumulation has also been shown to be involved in the regulation of cell death (Rao and Davis, 1999). Treatment of Arabidopsis with exogenous methyl jasmonate after an O_3 exposure decreased O_3-induced foliar lesion formation significantly (Overmyer et al., 2000), and jasmonic acid (JA) seems to be an important regulator in the lesion containment (Overmyer et al., 2000; Rao et al., 2000). In trees, an O_3-sensitive hybrid poplar (*Populus maximowizii* × *P. trichocarpa*) clone was shown to have a defect in both JA- and SA-signaling pathways and thus failed to trigger the SA- and JA-dependent defense responses, which were proposed to be the basis of its O_3 sensitivity (Koch et al., 1998, 2000). However, the role of ET in the O_3 sensitivity/tolerance in these hybrid poplars was not investigated. Therefore, we studied the significance of ET signaling on O_3-induced cell death in hybrid aspen (*Populus tremula* L. × *P. tremuloides* Michx.).

Chronic O_3 typically decreases net photosynthesis and accelerates foliar senescence in trees (Pell et al., 1999). However, it is not well understood how plants acclimate to chronic O_3 stress. Furthermore, the impact of sudden O_3 peaks in natural environments with elevated background O_3 is also mostly unex-

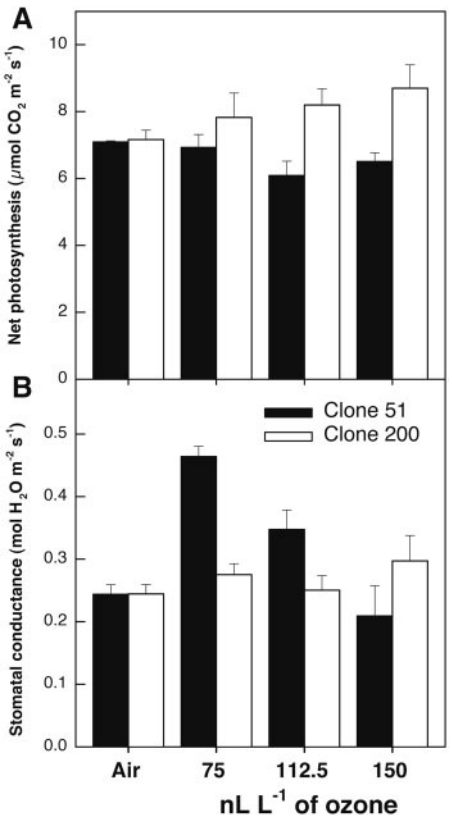

Figure 2. Net photosynthesis (A) and stomatal conductance (B) in hybrid aspen clones 51 and 200 in response to a single 8-h pulse of 75, 112.5, or 150 nL L^{-1} O_3. Photosynthesis was measured from the second and fourth fully expanded leaves after 5 to 6 h from the onset of the O_3 exposure. Error bars indicate ±SE ($n = 3$).

plored. We have elucidated the roles of ET, JA, and SA in the acclimation to O_3 in hybrid aspen clones that differ in their O_3 sensitivity. We show here that under continuous O_3 fumigation, blocking of ET perception with MCP protected leaves from accelerated senescence. In contrast, in the O_3-sensitive clone predisposed to chronic O_3 fumigation, an acute O_3 elevation caused severe necrosis when ET perception was blocked.

RESULTS

Responses under Acute, Single 8-h O_3 Pulse

To study the effects of an acute, short-term O_3 exposure on ET biosynthesis, the O_3-sensitive hybrid aspen clone 51 and the tolerant clone 200 were exposed to 75, 112.5, and 150 nL L^{-1} O_3 for 8 h. O_3 caused a clear concentration-dependent response in ET evolution and an increase in the *PT-ACS1* and *PT-ACS2* transcript levels (Fig. 1). At the concentration of 75 nL L^{-1} O_3, a tendency for increased ET production was observed in clone 51 when compared with clone 200 (two-way ANOVA, $F_{1,16} = 4.330$, $P = 0.054$; Fig. 1A). However, at the two higher concentrations, clones did not differ in ET production (Fig. 1A) or in the increase of ACS transcript abundance (Fig. 1B). At 75 nL L^{-1}, clone 51 had a slightly increased ion leakage, whereas clone 200 showed no response. However, at the two higher concentrations, 112.5 and 150 nL L^{-1} O_3, clone 200 had higher ion leakage than clone 51 (two-way ANOVA; $F_{1,16} = 10.144$, $P = 0.006$ and $F_{1,16} = 12.518$, $P = 0.003$, respectively; Fig. 1C). Despite the low level of ion leakage, none of the O_3 concentrations used in the acute treatments caused visible damage on the leaves of the two clones (data not shown), thus the clones did not differ in lesion formation under a short-term O_3 exposure.

Elevated O_3 did not decrease net photosynthesis in either of the clones in the acute 1-d exposure (Fig. 2A). However, a two-way ANOVA indicated that net photosynthesis was higher in clone 200 than in clone 51 at the higher O_3 concentrations used ($F_{1,16} = 16.067$, $P = 0.001$). As a response to a single 8-h exposure to 75 nL L^{-1} O_3, but not at the higher concentrations, stomatal conductance increased in clone 51 (one-way ANOVA, Dunnett's two-sided test, $P = 0.003$; Fig. 2B). In clone 200, stomatal conductance was not affected by any of the O_3 concentrations used (one-way ANOVA, $P = 0.498$; Fig. 2B).

Responses under Continuous Chronic O_3 Exposure

To study how ET affects photosynthetic activity during a continuous 19-d chronic O_3 exposure (75 nL L^{-1}), net photosynthesis and stomatal conductance were measured in the clean-air controls and in MCP-, O_3-, and MCP/O_3-treated leaves. O_3 decreased net photosynthesis in both clones (Fig. 3A; Table I). Blocking of ET perception with MCP delayed the decrease in net photosynthesis, which occurred during the normal foliar senescence, and abolished the effect of O_3 during the 19-d experiment (Fig. 3A; Table I). In contrast to the single, acute 8-h O_3 pulse of 75 nL L^{-1}, which increased stomatal conductance in clone 51 (Fig. 2), during the chronic O_3 treatment, stomatal conductance in clone 51 had a tendency to decrease. O_3 did not affect stomatal conductance in clone 200 (Fig. 3B; Table I), and blocking of ET perception with MCP did not affect stomatal conductance in either of the clones.

Chronic 3-week O_3 (75 nL L^{-1}) fumigation accelerated leaf senescence in O_3-sensitive clone 51. This was visible as a minor lesion formation and chlorosis (Fig. 4). Blocking of ET perception with MCP abolished the visible lesion formation, although the leaves still displayed chlorotic alterations (Fig. 4A) when compared with the clean-air controls and MCP-treated leaves (Fig. 4B). In the O_3-tolerant clone

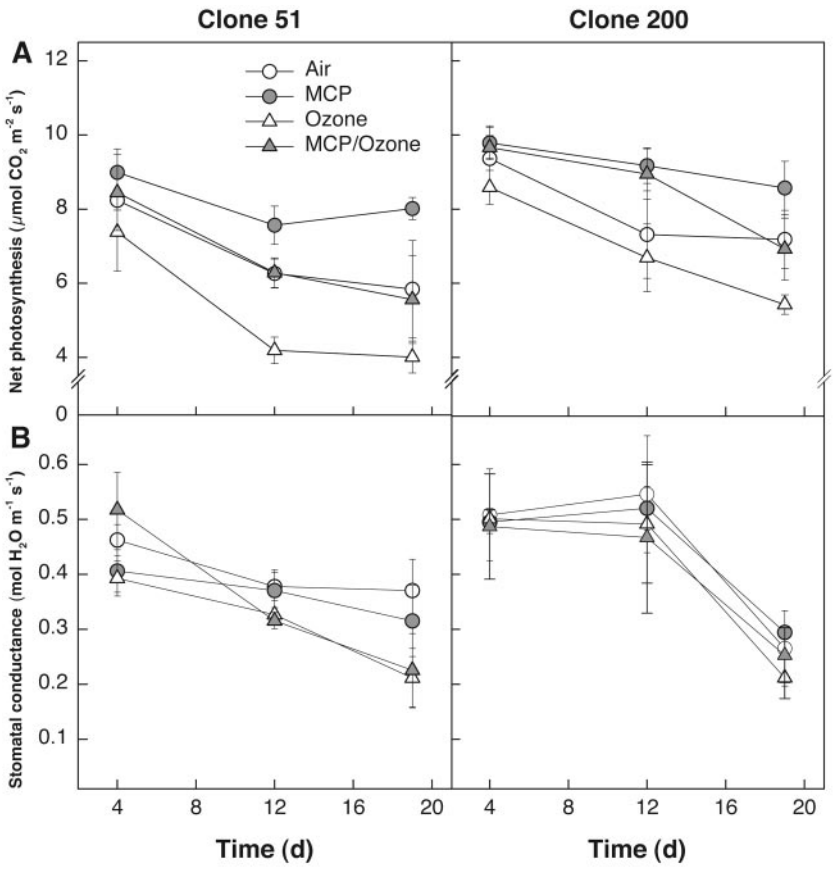

Figure 3. The effects of ET signaling on net photosynthesis (A) and stomatal conductance (B) in hybrid aspen clones 51 and 200 in response to chronic O_3 fumigation. Ramets were grown in clean air or treated with MCP, O_3, or MCP/O_3. ET receptors were blocked with 300 nL L^{-1} MCP in a sealed growth chamber. Chronic, low O_3 treatment was 75 nL L^{-1} O_3 for 19 d. Photosynthesis was measured from the second and fourth fully expanded leaves after 5 to 8 h from the onset of the daily O_3 exposure. Measurements were conducted on d 4, 12, and 19. Error bars indicate ±SE ($n = 3$). The main effects and interactions of sampling time, MCP, and O_3 are presented with an ANOVA in Table I.

200, no damage was apparent in either O_3 or MCP/O_3-treated leaves (data not shown).

Responses upon Acute O_3 Elevation

To simulate the transient O_3 episodes that are frequently observed in natural environments, one-half of the plants were subjected to an acute 1.5-fold O_3 elevation (from 75 to 112.5 nL L^{-1}) after 9 d of chronic O_3. This acute O_3 elevation caused the formation of visible lesions in the leaves of clone 51. When ET perception was blocked with MCP, O_3 elevation caused significantly more severe lesion formation in clone 51 when compared with the leaves exposed to O_3 only (Fig. 4C). The lesions in clone 51 occurred mainly in leaves 5 to 8 from the first fully emerged leaf. Clone 200 did not develop lesions in either O_3 or MCP/O_3-treated leaves, even after 9 d of elevated O_3 (Fig. 4D).

To elucidate whether acute O_3 elevation caused cell death in clone 51 through the exhaustion of antioxidant capacity, changes in the cellular redox balance were measured by analyzing the concentrations of oxidized glutathione (GSSG) and reduced glutathione (GSH) from leaves collected 8 h after the acute O_3 elevation when the lesion formation was becoming visible. The acute 1.5-fold O_3 elevation caused a rapid increase in the synthesis of glutathione in both clones (three-way ANOVA, $F_{1,16} = 87.822$, $P < 0.0005$; Fig. 5A). However, neither the total glutathione concentration nor the glutathione redox status differed between the two clones (three-way ANOVA, $F_{1,16} = 0.548$, $P = 0.470$ and $F_{1,16} = 0.363$, $P = 0.555$, respectively; Fig. 5, A and B). Similarly, on d 12, total glutathione concentration and glutathione redox status did not differ between the clones (data not shown). It can be concluded that O_3 lesion formation in clone 51 was not due to a reduced or insufficient antioxidative capacity that would be visible as changes in the cellular redox balance.

To evaluate the hormonal responses of the trees to chronic O_3 exposure and the effects of acute O_3 elevation on cell death, ET, JA, and SA were analyzed from leaf samples. O_3 increased ET production more in clone 51 than in clone 200 (Fig. 6A). This is also indicated by a significant clone × O_3 interaction in ANOVA (Table II). On d 12, the high O_3-induced ET evolution continued in clone 51, whereas clone 200 showed a decrease in ET evolution (Fig. 6A). The magnitude of ET production was consistent with *PT-ACS1* and *PT-ACS2* transcript accumulation (data not shown).

Free JA concentrations did not differ between the clean-air-grown clones (Fig. 6B). On d 10, O_3 elevation caused a slight increase in JA accumulation in clone 51, whereas in clone 200, no change was seen (Fig. 6B).

Table I. *ANOVA, effect of sampling time, MCP, and O_3 treatment on net photosynthesis and stomatal conductance of hybrid aspen clones 51 and 200 under chronic O_3 exposure (75 nL L^{-1}) for 19 d*

DF, Degrees of freedom.

Source	DF	Clone 51		Clone 200	
		F Value	*P*	*F* Value	*P*
Net photosynthesis					
Day	2	12.590	<0.0005	10.727	<0.0005
MCP	1	11.835	0.002	11.865	0.002
O_3	1	12.111	0.002	4.379	0.047
Day × MCP	2	0.460	0.637	0.853	0.439
Day × O_3	2	0.963	0.396	1.047	0.366
MCP × O_3	1	0.037	0.850	0.224	0.640
Day × MCP × O_3	2	0.231	0.795	0.036	0.964
Error	24				
Stomatal conductance					
Day	2	12.975	<0.0005	14.040	<0.0005
MCP	1	0.003	0.955	0.001	0.980
O_3	1	3.910	0.060	0.687	0.415
Day × MCP	2	0.397	0.676	0.177	0.839
Day × O_3	2	2.510	0.102	0.109	0.897
MCP × O_3	1	2.418	0.133	0.002	0.965
Day × MCP × O_3	2	1.033	0.371	0.002	0.998
Error	24				

The increased O_3-induced JA accumulation in clone 51 was also indicated as a significant clone × O_3 interaction during the experimental period (Table II).

The basal concentration of free SA was lower in the O_3-sensitive clone 51 than in the tolerant clone 200 (Fig. 6C). Moreover, whereas the free SA concentration remained constant throughout the experiment in the clean-air-grown clone 51, the O_3-tolerant clone 200 showed a progressive buildup of free SA during the experimental period as the leaves aged (Fig. 6C). The acute O_3 elevation on d 10 resulted in increased accumulation of free SA in the O_3-sensitive clone 51 when compared with the clean-air-grown ramets, whereas clone 200 was practically unaffected (Fig. 6C). On d 12, free SA concentrations increased in both clones (Fig. 6C). During the experimental period, the differential accumulation of free SA in these clones was also visible as a significant clone × day × O_3 interaction (Table II). Blocking of ET perception with MCP did not affect SA concentrations in either of the clones (Table II). Taken together, the O_3-sensitive clone 51 responded to acute O_3 elevation with high ET evolution and a late increase in SA concentration, whereas the O_3-tolerant clone 200 displayed a progressive buildup of SA and low O_3-induced ET.

DISCUSSION

ET Accelerates Leaf Senescence under Chronic O_3

Chronic elevated O_3 caused a differential response in the hybrid aspen clones. This was highly correlated and also mechanistically linked to ET evolution. The clones, however, did not vary in their responses to a single, acute 8-h pulse of increasing concentrations of O_3, which suggests that the difference between the clones is in their ability to acclimate to chronic O_3 exposure.

The stomatal aperture can be one of the factors that can determine plant O_3 sensitivity. For example, it has been shown that O_3 exposure reduced stomatal conductance in an O_3-tolerant hybrid poplar clone, which suggests that the entry of O_3 into the intercellular airspace was reduced (Koch et al., 1998). However, differential stomatal closure is not necessarily always the basis of O_3 sensitivity; in another example, Coleman et al. (1995) did not find consistently large differences in stomatal conductance in response to chronic O_3 stress among three trembling aspen (*Populus tremuloides*) clones that differed in their O_3 sensitivity. In the O_3-sensitive hybrid aspen clone 51 studied here, stomatal conductance increased upon the single 8-h pulse of low O_3 (75 nL L^{-1}) but not at higher concentrations. This increased stomatal conductivity, however, was not maintained because the clone 51 actually had lower stomatal conductance than the tolerant clone 200 after 4 d of exposure to chronic O_3. Thus under chronic O_3 exposure, the access of O_3 to the leaf cannot be regarded as the basis for the difference in the O_3 sensitivity between the clones.

Similarly, leaf antioxidant status can be one of the factors that affect the degree of plant O_3 sensitivity. For example, Sheng et al. (1997) proposed that glutathione plays an integral role in determining O_3 tolerance in three trembling aspen clones exposed to chronic O_3. In this study, the ratio of GSSG and GSH was similar in both clones after the acute increase in O_3 concentration, when lesion formation was evident only in the sensitive clone. Altogether these results suggest that the difference in the sensitivity and le-

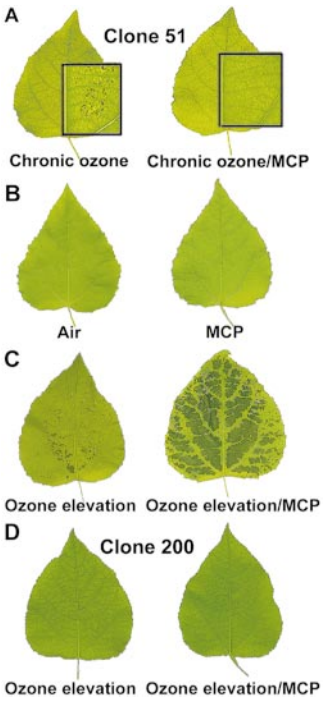

Figure 4. The effect of ET signaling on visual damage in response to continuous chronic O_3 fumigation in clone 51 and after an acute O_3 elevation in hybrid aspen clones 51 and 200. Chronic O_3 (75 nL L^{-1}) treatment was for 19 d. Acute, 1.5-fold O_3 treatment was conducted by subjecting ramets first for 9 d to chronic O_3 fumigation, after which O_3 concentration was increased 1.5-fold. ET receptors were blocked with 300 nL L^{-1} MCP. A, Visible alterations in clone 51 exposed to continuous chronic O_3 fumigation without and with MCP treatment photographed on d 19. Insets display magnified detail of leaves. B, Clean-air control and MCP-treated leaves of clone 51 photographed on d 19. C, Visible damage in clone 51 after the acute 1.5-fold O_3 elevation without and with MCP treatment photographed on d 11. D, Leaves of clone 200 after 9 d of acute 1.5-fold O_3 elevation with and without MCP treatment photographed on d 19.

sion formation between the clones used here is not based on higher entry of O_3 to the leaf and subsequent exhaustion of antioxidative capacity, but there must be other reasons for their differences in O_3 sensitivity under the chronic exposure.

In the O_3-sensitive clone 51, accelerated leaf senescence was observed as a response to chronic O_3 exposure; net photosynthesis was decreased, and minor lesion formation was evident. ET was required for both of these processes because they were prevented by blocking of ET perception with MCP. In the O_3-tolerant clone 200, chronic O_3 exposure decreased net photosynthesis, and also here, MCP prevented the ET-dependent decrease. This suggests that the O_3-induced ET was most likely involved in depressing net photosynthesis and promoting visible lesion formation. ET was not, however, required for the promotion of O_3-dependent chlorosis because MCP did not affect this process. The blocking of ET perception in the absence of O_3 also prevented the progression of normal senescence in both clones during the 3-week experiment. Pell et al. (1997) have proposed two alternative roles for O_3-induced ET in foliar senescence: Either ET can be regarded as an essential factor in the induction of accelerated leaf senescence, or it may serve as a facilitator of O_3-induced senescence and enhance the naturally occurring process. Because blocking of ET perception protected the leaves from the O_3-induced lesion formation and decline in net photosynthesis, ET presumably serves as a facilitator of O_3-induced senescence, as proposed by Pell et al. (1997).

Progressive Buildup of SA May Antagonize ET and Confer O_3 Tolerance in Hybrid Aspen

It has been shown that SA can inhibit ET biosynthesis in pear (*Pyrus communis*) cell suspension cultures (Leslie and Romani, 1988), in tomato fruits (Li et al., 1992), in rice (*Oryza sativa*) leaves (Huang et al., 1993), and in mung bean (*Vigna radiata*) hypocotyls (Lee et al., 1999). Because SA concentrations were markedly higher in the O_3-tolerant clone 200 than in

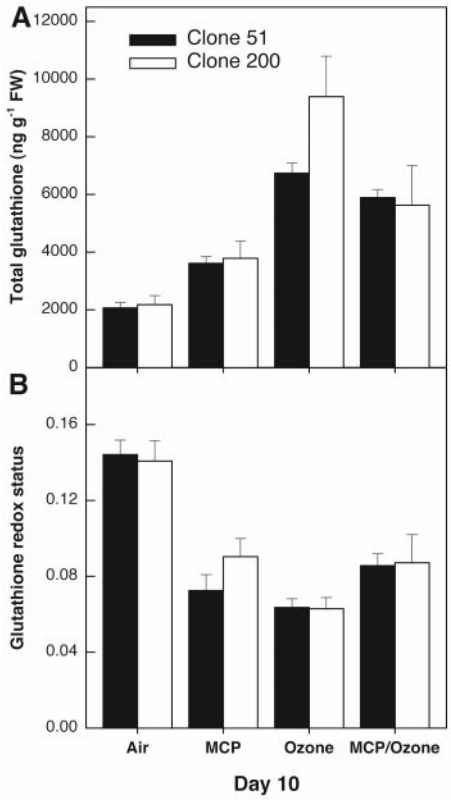

Figure 5. The concentration of total glutathione (A) and glutathione redox status (B) in hybrid aspen clones 51 and 200 in clean-air-grown and MCP, O_3, and MCP/O_3-treated leaves for 10 d. O_3 treatment was 75 nL L^{-1} O_3 for 9 d, followed by a 1.5-fold elevation in O_3 concentration. ET receptors were blocked with 300 nL L^{-1} MCP. Glutathione levels were measured 8 h after the acute O_3 elevation from the second and fourth fully expanded leaves by HPLC. Error bars indicate $\pm$SE ($n = 3$). Glutathione redox status was calculated as GSSG/(GSH + GSSG).

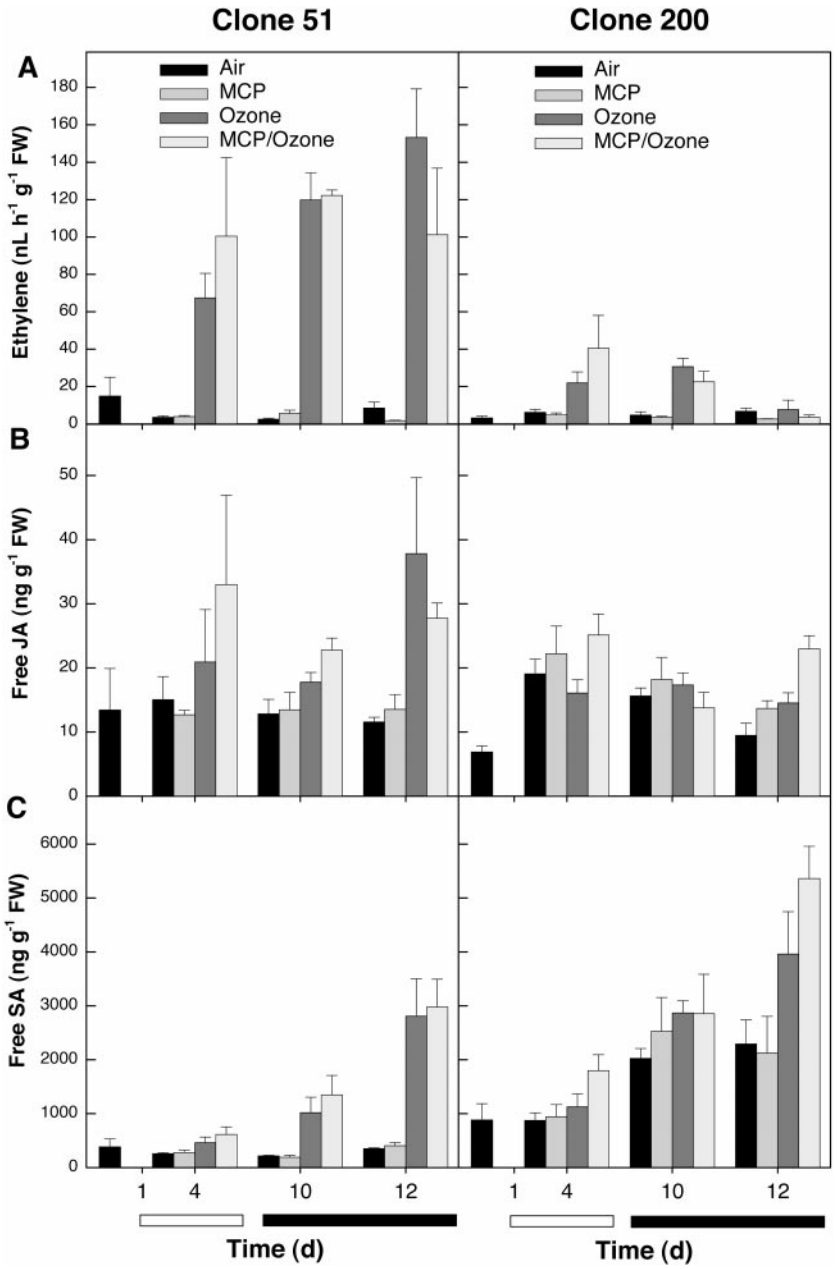

Figure 6. ET (A), JA (B), and SA (C) in hybrid aspen clones 51 and 200 in clean-air-grown and MCP, O_3, and MCP/O_3-treated leaves. O_3 treatment was 75 nL L^{-1} O_3 for 9 d, followed by an acute 1.5-fold O_3 elevation on d 10. ET receptors were blocked with 300 nL L^{-1} MCP. On d 1, 4, 10, and 12, the first and third fully expanded leaves were collected for ET evolution measurement and the second and fourth for JA and SA determinations. Error bars indicate ±SE. White bar on the bottom indicates 75 nL L^{-1} O_3, and black bar indicates the acute 1.5-fold O_3 elevation. The main effects and significant interactions of clone, sampling time, MCP, and O_3 are presented with an ANOVA in Table II.

the O_3-sensitive clone 51, SA might have depressed ET production in clone 200. Koch et al. (1998, 2000) proposed that the O_3-sensitive hybrid poplar clone NE-388 had a defect in SA signaling. This was proposed to be the basis of its O_3 sensitivity. However, this does not exclude the possibility that the O_3 sensitivity of this hybrid poplar clone could also be ET dependent, because it is also possible that the apparent SA insensitivity has prevented the down-regulation of ET synthesis by SA.

Accumulation of free SA increased in the O_3-sensitive hybrid aspen clone 51 after the O_3 elevation. This may be associated with acclimation to oxidative stress because no further lesion development was observed after the initial increase in O_3 concentration, which also induced the free SA concentration in clone 51 to increase to the same level as in clone 200. Therefore, as clone 200 displayed a progressive buildup of SA over the experimental period, SA might have served as a predominant protective signal with an essential role in the acclimation to oxidative stress. In addition, SA may also have prevented the up-regulation of ET synthesis and thus also the ET-dependent lesion formation in clone 200.

Highly Induced ET Biosynthesis and Simultaneous ET Insensitivity May Have Adverse Effects

Blocking of ET perception with MCP protected the leaves of the sensitive clone 51 from the minor lesion

Table II. *ANOVA, the main effects, and significant interactions of clone, sampling time, MCP, and O_3 on the accumulation of ethylene, jasmonic acid, and salicylic acid in hybrid aspen clones 51 and 200 under the chronic O_3 exposure (75 nL L^{-1}) for 4 d and upon a sudden 1.5-fold O_3 elevation on d 10*

DF, Degrees of freedom.

Source	DF	*F* Value	*P*
Ethylene			
Clone	1	55.554	<0.0005
Day	2	4.289	0.019
MCP	1	2.359	0.131
O_3	1	314.424	<0.0005
Clone × day	2	8.946	<0.0005
Day × MCP	2	4.991	0.011
Clone × O_3	1	74.579	<0.0005
Day × O_3	2	5.164	0.009
Clone × day × O_3	2	6.724	0.003
Error	48		
Jasmonic acid			
Clone	1	0.646	0.426
Day	2	1.507	0.232
MCP	1	3.826	0.056
O_3	1	23.530	<0.0005
Clone × day	2	2.857	0.067
Clone × O_3	1	10.474	0.002
Day × O_3	2	4.284	0.019
Error	48		
Salicylic acid			
Clone	1	251.329	<0.0005
Day	2	53.109	<0.0005
MCP	1	2.458	0.123
O_3	1	142.103	<0.0005
Clone × day	2	5.285	0.008
Clone × O_3	1	34.395	<0.0005
Day × O_3	2	8.905	0.001
Clone × day × O_3	2	5.702	0.006
Error	48		

formation during the chronic 9-d exposure, whereas the acute O_3 elevation induced severe necrotic cell death in the MCP-treated individuals (Fig. 4). This suggests that there are at least two distinct processes involved and that ET can have opposite roles in plant-O_3 relationships. Three alternative hypotheses can be suggested for the role of ET here. First, clone 51 may not have been able to trigger some ET-dependent responses necessary to counter elevated O_3 challenge. This may have prevented the up-regulation of defense responses required to survive oxidative stress. Second, it has been proposed (Mehlhorn and Wellburn, 1987) that ET may react with O_3 and form water-soluble, highly reactive ROS that eventually cause unregulated cell death. However, it has been shown in several species that O_3-induced cell death requires ET signaling (Bae et al., 1996; Tuomainen et al., 1997; Overmyer et al., 2000; Moeder et al., 2002), which argues against the hypothesis of O_3 and ET reacting together. Therefore, it is also unlikely that a reaction between ET and O_3 could have promoted cell death in hybrid aspen. Third, because ET biosynthesis is functional even when ET perception is inhibited, ET biosynthesis might have an indirect adverse effect that causes uncontrolled necrotic cell death. The conversion of ACC into ET has a highly toxic by-product, HCN, which may have been involved in cell death. Normally, plant tissues have an adequate capacity to detoxify HCN with β-CAS (Yip and Yang, 1988). However, as suggested by Grossmann (1996), under circumstances where ET biosynthesis is strongly stimulated, elevated HCN content may exceed the detoxification capacity and may result in necrotic cell death. According to results in birch (*Betula pendula* Roth; Vahala et al., 2003), induction of *β-CAS* requires functional ET perception and signaling. Thus it is plausible that also in hybrid aspen, the induction of *β-CAS* can be dependent on functional ET signaling and that HCN removal was not induced, which results in HCN-dependent cell death.

CONCLUSIONS

The negative effects of tropospheric O_3 are well known. Further increases in ambient O_3 concentrations, combined with acute O_3 episodes, may result in harmful effects for certain O_3-sensitive plant genotypes. Our results show that both hybrid aspen clones produced ET similarly under a short-term acute O_3 exposure, but the O_3-tolerant clone 200 was capable of acclimating under the chronic oxidative stress by keeping ET biosynthesis at a low level; instead, it accumulated high amounts of SA, which may possibly have attenuated the ET-dependent processes. Furthermore, ET signaling did not seem to be involved in the tolerance of clone 200 to O_3, but it was essential in clone 51 to prevent massive lesion formation upon acute O_3 elevation. ET signaling is involved in the decrease of net photosynthetic capacity under chronic O_3 fumigation, which indicates an apparent connection between photosynthesis and ET. Taken together, our results suggest that the O_3-sensitive clone 51 employed ET-dependent defenses, which may have a crucial role in O_3 responses, whereas in the O_3-tolerant clone 200 SA-dependent defenses may have a more central role.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Two hybrid aspen (*Populus tremula* L. × *P. tremuloides* Michx.) clones, 51 and 200, were selected based on preliminary results on their O_3 sensitivity. Clones were propagated by in vitro tissue culture, and copies of the clones (ramets) were planted and grown in peat:sand:vermiculite (6:1:1, w/v) under greenhouse conditions for 2 months. Ramets were fertilized with 1′89 nitrogen:phosphorus:potassium (11:4:25) every 2nd d. Ramets were transferred into growth chambers (a photoperiod of 22 h of light/2 h of dark, light intensity of 300 μmol m^{-2} s^{-1}, temperature of 19°C/12°C [light/dark], and relative humidity of 60%/70% [light/dark]), were allowed to acclimate at least for 4 d before the treatments, and were rotated within and among the chambers frequently. Ramets were tagged from the first fully expanded leaf from apex, which was designated as leaf 1.

O_3 and Chemical Treatments

The O_3 exposures were conducted in growth chambers. O_3 was produced from pure O_2 by electric discharge, and the delivery of O_3 to the chambers was computer-controlled based on continuous measurements of O_3 concentration inside the chamber with a Dasibi 1008-RS O_3 analyzer (Amko Systems Inc., Ontario, Canada) calibrated annually according to the instructions of manufacturer. To explore dose responses, the hybrid aspen clones were subjected to single 8-h O_3 pulses of 75, 112.5, or 150 nL L^{-1}. In the separate chronic O_3 exposures, hybrid aspen clones were exposed for 8 h to O_3 (75 nL L^{-1} from 10 AM to 6 PM) for 9 d, after which one-half of the population was subjected to an acute 1.5-fold O_3 elevation (112.5 nL L^{-1}) for 3 or 9 d.

ET receptors were blocked with 1-MCP (EthylBloc, Laboratorium Van der Sprong bv, AB Roelofarendsveen, Netherlands) in a sealed growth chamber with 300 nL L^{-1} MCP for 12 h according to the manufacturer's instructions. This treatment was performed daily or every 2nd d during the chronic O_3 experiment before the exposure to ensure the blocking of newly synthesized ET receptors. After each MCP treatment, the chamber was flow-through ventilated for 2 h before the onset of O_3 exposure to avoid any possible chemical reactions between O_3 and MCP.

Photosynthesis Measurements

The net photosynthesis, stomatal conductance, and transpiration measurements were performed at 5 to 8 h after the onset of O_3 exposure with LI-6400 photosynthesis system (LI-COR, Lincoln, NE). Leaves 2 and 4 were measured, and the mean value of the two leaves was calculated. Measurements were conducted from at least three plants per clone per treatment at 3 to 6 PM. All the measurements were made under 6400-02B red/blue LED light source with photon flux density of 1,000 μmol m^{-2} s^{-1} photosynthetically active radiation, temperature of 22°C, constant input of CO_2 (400 μL L^{-1}) from the minicartridges, constant air flow rate of 500 μmol s^{-1}, and relative humidity of 40% to 50%.

ET, Ion Leakage, JA, SA, and Glutathione Determinations

For ET determination, the first and third fully expanded leaves were collected in the single 8-h O_3 pulse experiments at 0, 2, 6, 8, and 24 h. In the chronic/acute O_3 experiments, leaves were sampled at 4 to 6 PM. Leaves were collected from three ramets for each time point. Leaves were sealed into 20-mL vials with 1 mL of tap water and incubated for 3 to 4 h at 22°C. ET was determined from a 1-mL air sample by flame ionizing gas chromatography (model 3700, Varian Medical Systems, Palo Alto, CA) with a porapak Q column (80–100 mesh, 1 m × 3.2 mm). Oven, injector, and detector temperatures were 40°C, 150°C, and 200°C, respectively. Cell death was quantified as ion leakage; after ET determination, the same leaves were transferred into 35 mL of 18.2 mOhm water and shaken 80 min at 300 rpm, and ion leakage was determined with a conductivity meter (Mettler Toledo GmbH, Greifensee, Switzerland). Total ion content was determined after killing the leaves by boiling. The final ion leakage is expressed as percentage of total ions.

For the JA and SA determinations, the second and fourth fully expanded leaves were collected in the chronic/acute O_3 experiments. Leaves were collected from three independent trees and were sampled at 4 to 6 PM on d 1, 4, 10, and 12. JA and SA were extracted and quantified essentially as described by Vahala et al. (2003).

Glutathione was extracted from the second and fourth fully expanded leaves in the chronic/acute O_3 experiments. Fifty milligrams of frozen leaves was powdered in liquid nitrogen, mixed with 1 mL of 2% (w/v) meta-phosphoric acid containing 1 mM EDTA and 1 mg polyvinylpolypyrrolidone mg^{-1} sample and centrifuged (20 min, 4°C, 30,000*g*). The supernatant was used for glutathione analyses. Glutathione was determined after reduction, derivatization with monobromobimanes, HPLC separation (Beckman System Gold, Beckman, Munich) on a C-18 column (Ultrasphere, Beckman Coulter, Fullerton, CA), and detection with a fluorescence detector (R_F-550, Shimadzu, Duisburg, Germany) as described (Schützendübel et al., 2001). The redox state is expressed as the GSSG/(GSH + GSSG) ratio.

Generation of ACS cDNA Probes and Northern Analysis

Leaves 2 and 4 were harvested for RNA extractions in the single 8-h O_3 pulse experiments at 0, 2, 6, 8, and 24 h, and in the chronic/acute O_3 experiments between 4 to 6 PM on d 1, 4, 10, and 12. Leaves were harvested from three ramets per treatment, frozen in liquid N_2, stored at −70°C, and pooled for RNA extractions. Total RNA was purified by lithium chloride precipitation as described (Chang et al., 1993). The hybrid aspen ACS cDNAs, *PT-ACS1* (AF518326) and *PT-ACS2* (AF518327) were isolated from in vitro-grown hybrid aspen leaves. *PT-ACS1* was isolated from total RNA of non-treated leaves, whereas *PT-ACS2* was isolated from RNA of leaves treated with 50 μM $CuCl_2$ for 6 h. The first strand of cDNA was synthesized by avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) using total RNA as the template, followed by PCR amplification with Dynazyme DNA polymerase (Finnzymes, Espoo, Finland) using the degenerate oligonucleotide primers *OLE-4*, *OLE-5*, and *OLE-6* (Botella et al., 1992). For northern analyses, total RNA was separated on a denaturing 1% (w/v) agarose gel in MOPS buffer and capillary blotted onto nylon membranes (Roche Diagnostics GmbH, Mannheim, Germany). Membranes were hybridized with probes prepared by High Prime labeling system (Roche Diagnostics) with [α-^{32}P]dCTP label and purified on G-50 columns (Amersham Biosciences, Piscataway, NJ). Prehybridizations, hybridizations, and high-stringency washes were performed at 68°C as described (Church and Gilbert, 1984). Hybridization signals were quantified with a phosphor imager and image analysis software (Bas 1500, Fujifilm, Tokyo). To normalize the loading of each individual lane in RNA gel, membranes were hybridized with a birch (*Betula pendula*) 18S rDNA probe.

Statistical Analyses

Factorial ANOVA was used to detect significant differences among clones and treatments. All data were checked for normality and heterogeneity of variances. Hormone and glutathione concentrations were $\log_{10}(X+1)$ transformed and glutathione redox-status values arcsin transformed to meet the assumptions of ANOVA. Data on net photosynthesis, stomatal conductance, and induction of ion leakage were used untransformed. Data on ion leakage were analyzed as an increase in ion leakage by subtracting the average value in clean-air-grown controls from O_3-induced values for each clone. One-way ANOVA, followed by Dunnett's two-tailed *t* test was used to detect significant differences between a control and treatments, where appropriate. Statistical analysis was carried out with SPSS v11.0 (SPSS Inc., Chicago).

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