Ethylene Insensitivity Modulates Ozone-Induced Cell Death in Birch[1]

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We have used genotypic variation in birch (*Betula pendula* Roth) to investigate the roles of ozone (O_3)-induced ethylene (ET), jasmonic acid, and salicylic acid in the regulation of tissue tolerance to O_3. Of these hormones, ET evolution correlated best with O_3-induced cell death. Disruption of ET perception by transformation of birch with the dominant negative mutant allele *etr1-1* of the Arabidopsis ET receptor gene *ETR1* or blocking of ET perception with 1-methylcyclopropene reduced but did not completely prevent the O_3-induced cell death, when inhibition of ET biosynthesis with aminooxyacetic acid completely abolished O_3 lesion formation. This suggests the presence of an ET-signaling-independent but ET biosynthesis-dependent component in the ET-mediated stimulation of cell death in O_3-exposed birch. Functional ET signaling was required for the O_3 induction of the gene encoding β-cyanoalanine synthase, which catalyzes detoxification of the cyanide formed during ET biosynthesis. The results suggest that functional ET signaling is required to protect birch from the O_3-induced cell death and that a decrease in ET sensitivity together with a simultaneous, high ET biosynthesis can potentially cause cell death through a deficient detoxification of cyanide.

The concentration of tropospheric ozone (O_3) has increased during the past decades due to human activities, and it has been estimated that in year 2100, 50% of global forest area will be exposed to potentially phytotoxic O_3 concentrations (Fowler et al., 1999). In the leaves of O_3-sensitive plants, symptoms of high O_3 are observed as rapid lesion formation. Traditionally, formation of reactive oxygen species (ROS), such as superoxide ($O_2\cdot^-$) and hydrogen peroxide (H_2O_2) from the degradation of O_3 in the apoplast has been thought to alter the integrity of the plasma membrane and thus the integrity of the cell (Laisk et al., 1989; Heath, 1994). However, O_3 also induces an active and controlled apoplastic oxidative burst, the production of $O_2\cdot^-$ and H_2O_2 in the leaves affected, which may initiate programmed cell death analogous to that induced by ROS in an incompatible plant-pathogen interaction (Schraudner et al., 1998; Pellinen et al., 1999, 2002; Overmyer et al., 2000; Moeder et al., 2002; Wohlgemuth et al., 2002).

Activation of ethylene (ET) biosynthesis by induction of the genes encoding 1-aminocyclopropane-1-carboxylate synthase (ACS) is one of the fastest and most obvious biochemical responses to O_3 and has been mechanistically linked to the regulation of O_3 lesion formation (Schlagnhaufer et al., 1997; Tuomainen et al., 1997; Vahala et al., 1998; Overmyer et al., 2000; Moeder et al., 2002). ET is perceived by a two-component His kinase receptor family (Chang et al., 1993a; Hua et al., 1995, 1998; Sakai et al., 1998). The ET receptor can be pharmacologically blocked with a competitive inhibitor of ET action, norbornadiene, or with 1-methylcyclopropene (MCP; Serek et al., 1995), which prevents the binding of ET to the receptor. The known dominant ET receptor mutations also prevent ET perception and thus cause ET insensitivity (Hall et al., 1999). This has been used to create stable ET insensitivity in petunia (*Petunia hybrida*), tomato (*Lycopersicon esculentum*), and tobacco (*Nicotiana tabacum*) by transforming them with the Arabidopsis *etr1-1* mutant allele (Wilkinson et al., 1997; Knoester et al., 1998).

Inhibition of ET biosynthesis with inhibitors of ACS has significantly reduced O_3-induced lesion formation in the leaves of O_3-exposed plants (Mehlhorn and Wellburn, 1987; Mehlhorn et al., 1991; Schlagnhaufer et al., 1995; Wenzel et al., 1995; Tuomainen et al., 1997; Moeder et al., 2002). Similarly, defective ET signaling in the ET-insensitive *ein2* mutant (Overmyer et al., 2000) or inhibition of ET per-

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ception with exogenous norbornadiene reduced O_3-induced cell death in tomato (Bae et al., 1996; Moeder et al., 2002) and the spread of O_3-induced programmed cell death in an O_3-sensitive Arabidopsis *radical induced cell death1* (*rcd1*) mutant (Overmyer et al., 2000). Furthermore, in tomato, both ET biosynthesis and the subsequent H_2O_2 accumulation, which was required for the O_3 lesion formation, were highly colocalized, and inhibition of ET synthesis or perception also significantly reduced H_2O_2 accumulation and O_3 lesion formation (Moeder et al., 2002). Taken together, in tomato and Arabidopsis, both ET synthesis and signaling are required in the processes that result in O_3 lesion formation.

In addition to ET, jasmonic acid (JA), and salicylic acid (SA) are involved in the regulation of oxidative stress responses in plants. JA is commonly regarded as a protective compound (Creelman and Mullet, 1995; Berger, 2001) and may have an essential role in the control of proper lesion containment upon pathogen and O_3 challenge (Thomma et al., 1998; Overmyer et al., 2000; Berger, 2001). SA is a central component in plant pathogen defense. It is involved in the regulation of the oxidative burst, cell death in hypersensitive response, defense signaling, and systemic acquired resistance (Godiard et al., 1994; Staskawicz et al., 1995; Draper, 1997; Shirasu et al., 1997; Ciardi et al., 2000; Dangl and Jones, 2001). SA has a dual role in the control of plant O_3 responses; SA promotes the oxidative cell death during a short-term O_3 challenge but is also required for the up-regulation of defenses during a long-term oxidative challenge (Rao and Davis, 1999, 2001).

The mechanisms of stress resistance and acclimation in trees are not well understood; for example, there are only a few studies on the hormonal interactions during oxidative stress in trees. In hybrid poplar (*Populus maximowizii* × *P. trichocarpa*), sensitivity to acute high O_3 correlated with deficiencies in both JA- and SA-signaling pathways (Koch et al., 1998, 2000). However, the role of ET was unexplored. We have explored the role of ET under low chronic O_3 in the accompanying study (Vahala et al., 2003), which shows that ET also modifies O_3 sensitivity in hybrid aspen. In the experiments reported here, we have characterized the involvement of all three signaling pathways, ET, JA, and SA, in cell death induced by a short exposure to high O_3 concentration in silver birch (*Betula pendula* Roth) genotypes differing in their O_3 sensitivity. The role of ET was elucidated in detail using transgenic ET-insensitive birch lines and inhibitors. We show that ET insensitivity in birch reduced but did not completely eliminate O_3-induced cell death when inhibition of ET biosynthesis abolished the lesion formation. This suggests the presence of an ET-signaling-independent but ET biosynthesis-dependent component in the ET-mediated stimulation of cell death in O_3-exposed birch.

RESULTS

Kinetics of O_3-Induced ET, JA, and SA Production among Three Wild-Type Birch Clones

Three birch clones, the O_3-tolerant E9702, the moderately sensitive K1898 and the sensitive V5818 were selected from 17 genotypes differing in their O_3 sensitivity. One-year-old copies of the clones (ramets) were exposed to 200 nL L^{-1} O_3 for 8 h. Over the whole experimental period, the clones did not differ statistically significantly in O_3-induced ion leakage but displayed a differential pattern in O_3-induced ion leakage (Fig. 1A).

ET evolution was highest in the O_3-sensitive V5818 and lowest in the tolerant E9702. ET evolution was maximal at 4 to 8 h and correlated with ion leakage in all three clones (Table I). All of the changes in ET evolution were caused by O_3 because ET production did not change in air-grown plants throughout the experiments (data not shown). O_3 induced a significant increase in the accumulation of JA at 8 h in the O_3-sensitive clone V5818, whereas only a slight increase was observed in K1898 and no increase was observed in the O_3-tolerant E9702 (Fig. 1C). SA accumulated in significant amounts only 24 h after the onset of O_3 exposure, especially in the O_3 tolerant clone E9702. Clone K1898 accumulated intermediate levels of SA, whereas no SA accumulation was evident in the O_3-sensitive V5818 (Fig. 1D). O_3-induced ion leakage, JA and SA concentrations, and ET evolution increased more rapidly in clone K1898 than in the two other clones.

Kinetics of Transcript Accumulation for Genes of ET Biosynthesis Correlates with O_3 Tolerance

Three birch ACS cDNAs were isolated to study whether the expression kinetics of ACS genes differed among the clones. In E9702, transcript levels for *BP-ACS1* (AY120897) and *BP-ACS2* (AY120898) reached the maximum at 4 h, whereas in V5818 and K1898, the maximum was reached at 8 h (Fig. 2, A and B). In V5818, the steady-state transcript levels for *BP-ACS1* and *BP-ACS2* were approximately 140- and 30-fold higher, respectively, than the initial level at 8 h, whereas in E9702, transcripts for *BP-ACS2* were almost undetectable at 8 h. *BP-ACS3* (AY120899) expression was not affected by O_3 (data not shown). In all clones, *BP-ACO1* (l-aminocyclopropane-l-carboxylate oxygenase; Y10749) transcript levels decreased between 0.5 and 1 h, increased back to the initial level at 2 h, and thereafter again decreased (Fig. 2C). The increase in transcript accumulation for *BP-ACO2* (AY154649) was among the fastest responses to O_3 and attained the maximum in clone E9702 at 2 h, and in clones K1898 and V5818 at 8 h (Fig. 2D).

Generation of Transgenic, ET-Insensitive Birch Lines

Of the hormones investigated, ET evolution had the highest correlation with O_3-induced cell death in all three clones (Table I). The role of ET in O_3-induced cell death was studied further with inhibitors of ACS and ET perception and by generating in five different genetic backgrounds several transgenic birch lines that carry the dominant *etr1-1* mutant allele of the Arabidopsis ET receptor gene *ETR1*. The degree of ET insensitivity in the transgenic lines was assessed by ET-induced leaf abscission and induction of an ET-regulated gene. After a 3-d exposure to ET, most of the wild type but none of the transgenic trees had freely abscised their leaves (Fig. 3D). When all of the remaining leaves were pulled with a rather strong force, a greatly attenuated leaf abscission was evident in 17 of the 23 independent transgenic lines examined (Fig. 3A). Southern analysis with the Arabidopsis *ETR1* revealed that the number of *etr1-1* inserts varied between the transgenic lines. For example, line 35 had two *etr1-1* inserts, and line 86 had one (Fig. 3B).

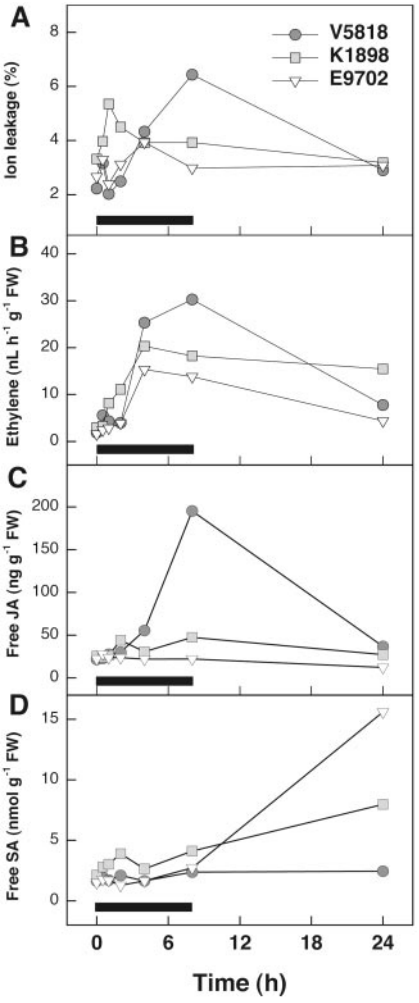

Figure 1. Ion leakage, ET evolution, and JA and SA concentrations in three 1-year-old wild-type birch clones, V5818, K1898, and E9702, in response to O_3. Clones were subjected to 200 nL L^{-1} O_3 for 8 h, and leaves were harvested for ion leakage and hormone determinations after the beginning of the exposure at the times indicated. Black bar indicates the duration of O_3 fumigation. Measurements were conducted from three independent ramets at each time point/clone. Two-way ANOVA (clone and time as factors) followed by Tukey's honestly significant difference (HSD) mean-separation test was used to detect statistically significant differences between the clones. A, Ion leakage ($P = 0.163$); B, ET evolution ($P = 0.074$); C, free JA concentrations (ANOVA, $P < 0.0005$; E9702 had lower JA accumulation than V5818 and K1898, Tukey's HSD mean-separation test $P < 0.0005$ and $P = 0.001$, respectively); D, free SA concentrations (ANOVA, $P < 0.0005$; K1898 had higher SA accumulation than V5818 and E9702, Tukey's HSD mean-separation test $P < 0.0005$ and $P = 0.006$, respectively).

Table I. *Correlation analysis of ion leakage, ET, JA, and SA in one-year-old clones V5818, K1898, and E9702 in response to O_3.*

The clones were subjected to 200 nL L^{-1} of O_3 for 8 h, and leaves were harvested for ion leakage and hormone determinations at 0, 0.5, 1, 2, 4, 8, and 24 h from the beginning of the O_3-exposure. Correlation analysis was conducted with the nonparametric Spearman's *p*. The statistical differences were considered significant at the level of $P < 0.05$. $^{**}P < 0.01$; $^{*}P < 0.05$.

Clone	Hormone	Ion Leakage	ET	JA
V5818	ET	0.643**		
	JA	0.552**	0.582**	
	SA	0.173	0.121	0.392
K1898	ET	0.477*		
	JA	0.512*	0.376	
	SA	−0.004	−0.016	0.371
E9702	ET	0.469*		
	JA	−0.053	−0.022	
	SA	0.110	0.438	−0.001

ET insensitivity was also visible as impaired ET induction of gene expression in the transgenic lines. A birch mitochondrial phosphate translocator gene (*BP-MPT1* [Y08499]; Kiiskinen et al., 1997) was strongly induced by ET in the wild-type birch clones but not in the transgenic lines. This was tested in five wild-type clones and 12 independent transgenic lines with similar results; the results for two of the transgenic lines (35 and 86) and the corresponding wild-type clone V5834 are shown in Figure 3C. We selected line 35, denoted hereafter as *BPetr1-1-35*, for further studies on the role of ET perception in O_3-induced cell death. The O_3 sensitivity of V5834 is similar to clone V5818 used in the other experiments (J. Vahala, H. Tuominen, and J. Kangasjärvi, unpublished data).

ET Insensitivity Reduces But Does Not Prevent O_3-Induced Cell Death

The ET-insensitive *BPetr1-1-35* had less O_3 damage (two-way ANOVA; genotype and time as factors; $P = 0.001$; Fig. 4A) but similar ET evolution (two-way ANOVA; $P = 0.944$; Fig. 4B) when compared with the wild-type clone V5834. The remaining O_3 lesions were mainly localized near the veins. Because the

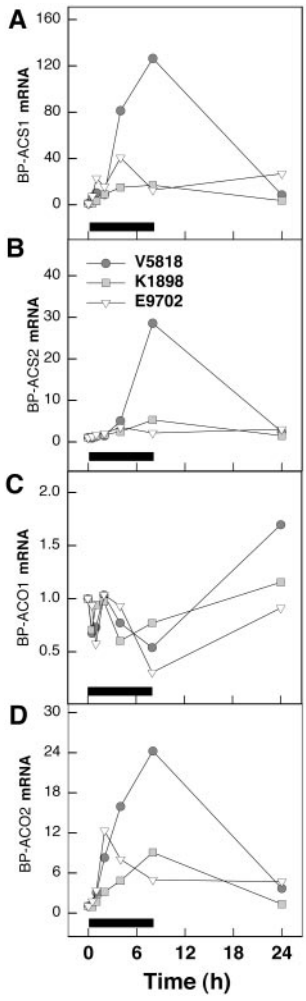

Figure 2. Relative increases in transcript abundance for the enzymes of ET biosynthesis in three 1-year-old wild-type birch clones, V5818, K1898, and E9702, in response to O_3. Clones were subjected to 200 nL L^{-1} O_3 for 8 h, and leaves for RNA isolation were harvested at the times indicated. Hybridization signals of each transcript are indicated as -fold increase of the initial level at 0 h. Black bars indicate the duration of O_3 fumigation. A, mRNA levels of *BP-ACS1*; B, mRNA levels of *BP-ACS2*; C, mRNA levels of *BP-ACO1*; and D, mRNA levels of *BP-ACO2*.

transgenic lines are insensitive to ET throughout their development and thus might lack some ET-dependent constitutive defenses, we elucidated the significance of ET signaling in the wild-type birch during a short-term O_3 exposure by blocking of ET perception with MCP only during the exposure. Similarly, as in the transgenic *BPetr1-1-35*, inhibition of ET perception in the wild-type clones partially reduced, but did not fully prevent visible O_3 lesion formation in the leaves of 1-year-old V5818 trees (Fig. 5A). Likewise, blocking of ET perception reduced the O_3-induced visible cell death in 3-month-old leaves of clone V5818 (Fig. 5B).

To demonstrate that the MCP that could potentially be remaining in the cells did not cause any unexpected effects (for example, by possibly reacting with O_3 during the exposure), we treated two wild-type birch clones, V5818 (Fig. 5C) and K1898 (not shown), with MCP immediately after a 6-h O_3 pulse. The blocking of ET perception with MCP after the O_3 treatment, again, only reduced cell death in both clones. This also indicates that ET is required for the O_3 lesion formation after the actual contact of O_3 with the apoplastic structures. In the transgenic *BPetr1-1-35*, O_3 damage was not reduced any further by a MCP treatment, whereas in the wild-type V5834, the extent of visual O_3 lesions was reduced by 32% (data not shown). Thus, the transformation of the genomic clone of the Arabidopsis *etr1-1* allele caused such a strong ET insensitivity in birch that it could not be further increased by exogenous MCP.

Inhibition of ET Biosynthesis Prevents O_3-Induced Cell Death

The ACS inhibitor AOA was used to determine how prevention of ET biosynthesis affects O_3 lesion formation in wild-type clone V5834 and the transgenic line *BPetr1-1-35*. AOA abolished ET production (three-way ANOVA; genotype, time and treatment as factors; Tukey's HSD mean-separation test; $P < 0.0005$) and prevented the O_3-dependent cell death completely in both wild-type and transgenic trees (Tukey's HSD mean-separation test; $P = 0.001$; Fig. 4, C and D). Similarly, AOA treatment of either 3-month-old (data not shown) or 1-year-old V5818 ramets abolished O_3-induced formation of visible lesions (Fig. 5D) and eliminated O_3-induced ion leakage (three-way ANOVA; genotype, time and treatment as factors; $P < 0.0005$) and ET evolution completely (three-way ANOVA; $P < 0.0005$; data not shown). For an unknown reason, treatment with Tween 20 alone also reduced slightly the O_3-induced ion leakage and ET evolution. Measured in wild-type clone V5818, the stomatal conductance in AOA-treated leaves was approximately 2-fold higher than either in O_3 or O_3/MCP-treated leaves (data not shown). Thus, differential O_3 lesion formation under MCP and AOA treatments was not based on higher entry of O_3 to the leaf.

The O_3-Induced Activation of β-Cyano-Ala Synthase (β-CAS) Is Dependent on ET

The results above suggest that the ET-dependent cell death is differentially affected by ET synthesis and signaling and prompts the question of what is the basis of cell death in the O_3-exposed ET-insensitive plants that can be prevented by inhibition of ET biosynthesis. The ACO-catalyzed oxidation of 1-aminocyclopropane-1-carboxylic acid (ACC) to ET produces an equimolar amount of cyanide (HCN), which is normally rapidly detoxified to β-cyano-Ala by β-CAS (Akopyan et al., 1975; Wurtele et al., 1985; Yip and Yang, 1988). We examined whether the birch

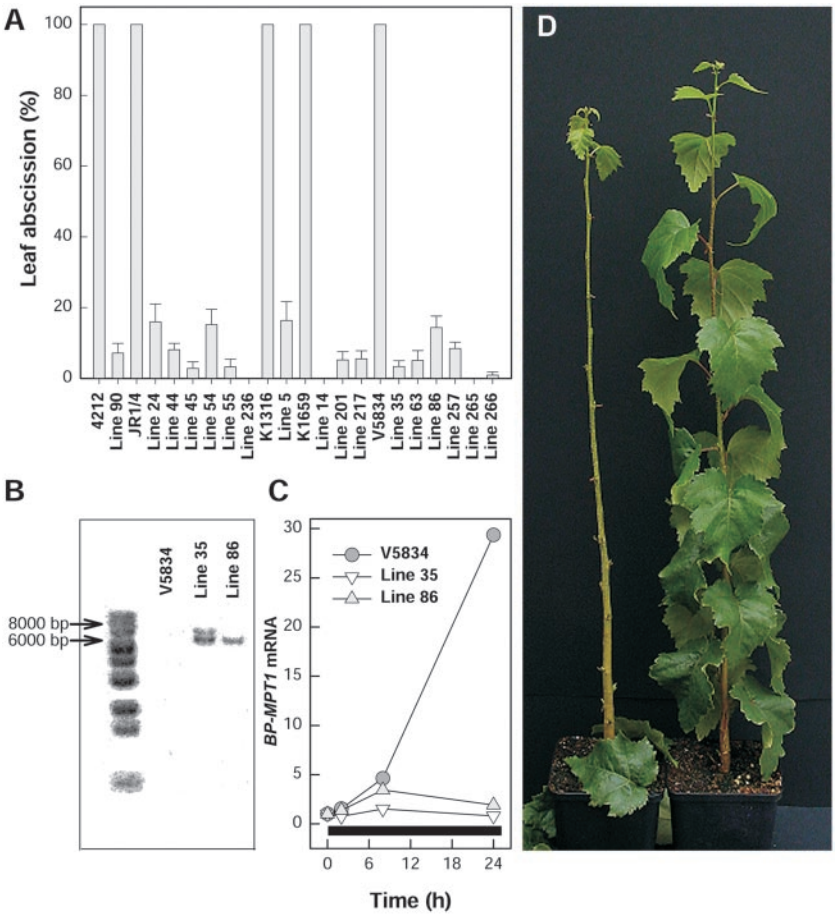

Figure 3. Evidence for ET insensitivity in the transgenic birch lines carrying Arabidopsis ET receptor gene with the dominant *etr1-1* mutation. A, Leaf abscission in response to 50 μL L^{-1} ET for 3 d in wild-type clones 4212, JR1/4, K1316, K1659, and V5834 and in 17 transgenic birch lines. Error bars indicate $\pm$SE (n = 6–11). Leaf abscission in each clone was assessed by pulling with similar force every leaf of the trees exposed to ET. B, Southern analysis for the presence of the Arabidopsis *etr1-1* gene in the wild-type clone V5834 and in transgenic birch lines 35 and 86. Birch genomic DNA was digested with *Kpn*I and was hybridized with an Arabidopsis *ETR1*-specific probe. C, Accumulation of birch mitochondrial phosphate translocator (*BP-MPT1*) transcript in the wild-type clone V5834 and in the transgenic birch lines 35 and 86 in response to 50 μL L^{-1} ET. Black bar indicates the duration of ET treatment. D, Leaf abscission in response to a 3-d ET treatment in the wild type (left) and *etr1-1* transformed transgenic line (right) of birch.

β-CAS gene (AY154650) is ET regulated. Results in Figure 6A show that when in the wild-type clone V5834 *β-CAS* transcript accumulation increased over 16-fold by exposure to ET, in the ET-insensitive *BPetr1-1-35* *β-CAS* was not induced. In response to O_3, *β-CAS* transcript accumulation was also lower in *BPetr1-1-35* than in the wild-type V5834 (Fig. 6B), whereas ET evolution was equal (Fig. 4B). Similarly, blocking of ET receptors with MCP reduced *β-CAS* transcript accumulation in response to O_3, but ET evolution was not affected (Fig. 6C). Thus, it can be concluded that functional ET signaling is required for the O_3-induced activation of *β-CAS* in birch and that as a result of decreased ET sensitivity, HCN detoxification can be compromised.

DISCUSSION

O_3-Induced Cell Death Correlates with ET and JA Accumulation

According to the recent results in several studies (Rao and Davis, 2001), plant responses to high concentrations of O_3 share common features with pathogen attack due to the function of ROS as signaling molecules. In this study, we employed high O_3 concentrations as a tool to probe the interactions of hormonal responses and the high level of ROS in birch. The results of these experiments address more the interactions and roles of SA, JA, and ET with ROS in the regulation of ROS-dependent cell death, and thus are not directly applicable to describe or represent plant responses to O_3 in natural environments. The latter we have evaluated in the accompanying paper (Vahala et al., 2003; this issue), where the roles of same hormones in response to chronic O_3 concentrations have been studied in hybrid aspen (*Populus tremula* × *P. tremuloides*) under more realistic levels of O_3 found in natural environments.

The timing and magnitude of ET evolution correlated with the extent of cell death in the wild-type birch clones. As could be expected, blocking of ET biosynthesis in the O_3-sensitive clone V5818 reduced O_3-induced cell death significantly, indicating the requirement for ET. The O_3-sensitive clone V5818 displayed high O_3-induced ET accumulation without the late SA accumulation, whereas the O_3-tolerant clone E9702 had low O_3-induced ET and highly induced late SA accumulation. This suggests that the early high ET production may antagonize the late SA accumulation, or vice versa, increased SA production may down-regulate ET accumulation and thus prevent the ET-dependent cell death.

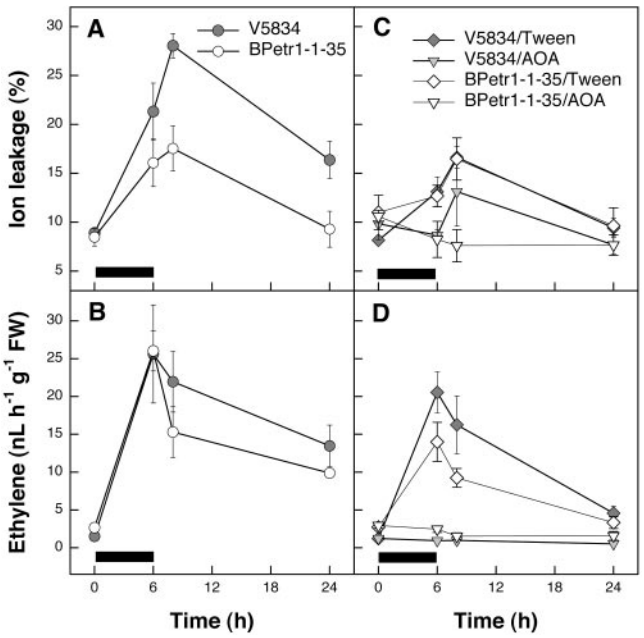

Figure 4. O_3-induced ion leakage, ET evolution, and the effect of blocking of ET biosynthesis in 3-month-old birch clone V5834 and in the transgenic line *BPetr1-1-35* carrying Arabidopsis ET receptor gene with the dominant *etr1-1* mutation. Ramets were sprayed either with 0.05% (v/v) Tween 20 or with 1.5 mM aminooxyacetic acid (AOA) in 0.05% (v/v) Tween 20 to block ET biosynthesis and were exposed to 200 nL L^{-1} O_3 for 6 h. Leaves were harvested for ET and ion leakage determinations at 0, 6, 8, and 24 h. Error bars indicate ±SE ($n = 3$). Black bars indicate the duration of O_3 exposure. A, Ion leakage in the wild-type clone V5834 and in the transgenic line *BPetr1-1-35*. B, ET evolution in the wild-type clone V5834 and in the transgenic line *BPetr1-1-35*. C, Ion leakage in the wild-type clone V5834 and in the transgenic line *BPetr1-1-35* with and without the AOA treatment. D, ET evolution in the wild-type clone V5834 and in the transgenic line *BPetr1-1-35* with and without the AOA treatment.

Similarly, the basal SA level of the ET-insensitive Arabidopsis mutant *ein2* was higher than in wild type, suggesting that ET can inhibit SA biosynthesis (H. Tuominen, K. Overmyer, M. Keinänen, and J. Kangasjärvi, unpublished data). The same interaction works also in the opposite direction; O_3-induced, EIN2-dependent gene expression was suppressed in the SA-deficient NahG plants. These results suggest similar regulation and interaction between ET and SA during oxidative stress in both Arabidopsis and birch. The role of JA is, however, more complicated. In birch, both ET and JA accumulation correlated with O_3-induced cell death in the O_3-sensitive clones V5818 and K1898, whereas in the O_3-tolerant clone E9702, JA concentration did not increase. Similarly, in the O_3-sensitive Arabidopsis mutants *rcd1* and *jar1*, high JA accumulation was evident. It has been shown that JA is involved in lesion containment (Overmyer et al., 2000), which at first may seem contradictory to the results of JA accumulation in the sensitive clones and not in the tolerant ones. However, because the biosynthesis of JA appears to be limited by substrate availability (Laudert et al., 2000; Ziegler et al., 2001), it is also possible that the accumulation of JA is a consequence of the ET-dependent cell death: Substrate for JA synthesis (α-linolenic acid or 13-(*S*)-hydroperoxylinolenic acid) could be released from the dying cells, thus resulting in in-

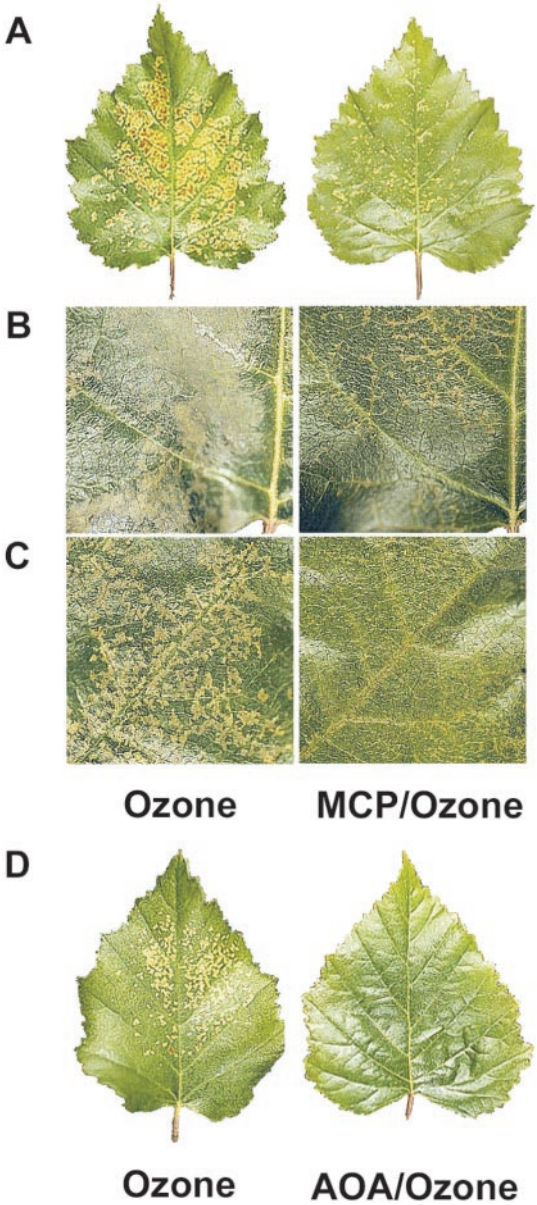

Figure 5. Visual damage in response to O_3 in birch after the inhibition of ET perception or biosynthesis. ET perception was blocked with 300 nL L^{-1} MCP and ET biosynthesis with 1 mM AOA. Ramets were exposed to 200 nL L^{-1} O_3 for 6 h. Photographs were taken 48 h after the beginning of the O_3 exposure. A, An O_3-treated leaf of the 1-year-old clone V5818 (left) and a leaf pretreated with MCP and then with O_3 (right). B, A detail of an O_3-treated leaf of the 3-month-old clone V5818 (left) and a leaf pretreated with MCP and then with O_3 (right). C, A detail of an O_3-treated leaf of the 3-month-old clone V5818 (left) and a leaf treated with MCP (right) after the O_3 pulse. D, An O_3-treated leaf of the 1-year-old clone V5818 (left) and a leaf treated with AOA and O_3 (right).

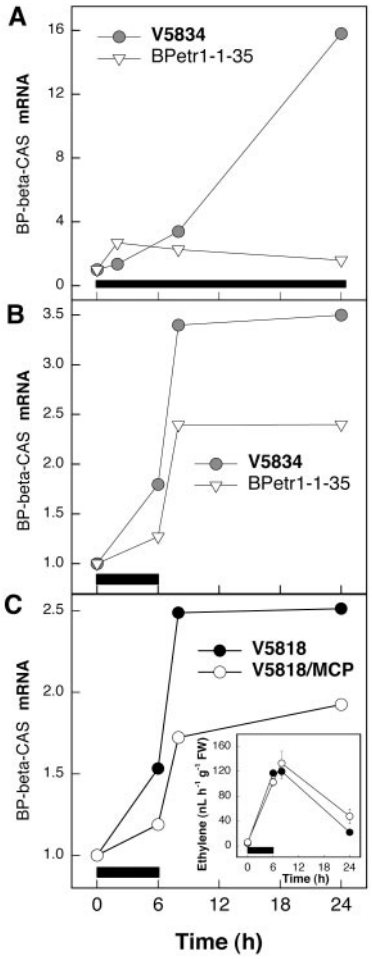

Figure 6. Accumulation of β-CAS transcript in response to ET or O_3. Birch clone V5834, the transgenic *BPetr1-1*-35 line carrying the Arabidopsis ET receptor gene with the dominant *etr1-1* mutation, and clone V5818 with blocked ET perception were exposed to O_3 or ET, and the transcript levels of β-CAS were analyzed. Ramets were subjected to 50 μL L^{-1} ET for 24 h or 200 nL L^{-1} O_3 for 6 h. ET perception was blocked with 300 nL L^{-1} MCP. Black bars indicate the duration of treatments. A, β-CAS mRNA accumulation in response to ET treatment in the wild-type clone V5834 and in the transgenic *BPetr1-1*-35 line. B, β-CAS mRNA accumulation in response to O_3 in the wild-type clone V5834 and in the transgenic line *BPetr1-1*-35. C β-CAS mRNA accumulation in response to O_3 in the control and MCP-treated wild-type clone V5818. Inset, ET evolution in the control and MCP-treated ramets in response to O_3.

creased JA synthesis, which then halts the ET-dependent lesion propagation.

A Dual Role for ET in O_3 Responses

ET can have opposite roles in O_3-exposed plants. Several reports have shown an essential pro-death role for ET in O_3-exposed plants. However, depending on the temporal pattern of biosynthesis ET may also have a pro-survival role. In mung bean (*Vigna radiata*) and pea (*Pisum sativum*), pretreatment of plants with ET before O_3 exposure promoted O_3 tolerance (Mehlhorn, 1990). Involvement of ET-dependent protection from oxidative stress may require a sufficient level of rapid but transient ET induction, however, at the intensity where it does not exceed the rate that induces prolonged spreading cell death. In the O_3-sensitive clone V5818, timing and degree of the late ET accumulation was most likely beyond the limit to provide protection against the O_3 challenge. This is also supported by the fact that blocking of ET perception after the O_3 exposure decreased O_3 damage, which indicates that the ET action for the promotion of cell death is required late. Although the early transcript accumulation of *BP-ACS1* was evident in all birch clones in response to O_3, *BP-ACS2* transcript levels increased considerably only in the O_3-sensitive clone V5818 at 8 h. Therefore, in these three wild-type clones, differential ACS transcript accumulation and ET production suggest a dual role for ET. Depending on the magnitude of synthesis and its temporal pattern, ET can serve as a mediator of either survival or cell death. The results presented here suggest that in the O_3-sensitive birch clone ET was required to promote cell death at the time of lesion development and that the requirement of ET, for example, for the induction of antioxidant defenses had less significance.

Transformation of Arabidopsis *etr1-1* Allele Causes Strong ET Insensitivity in Birch

The dominant Arabidopsis *etr1-1* mutation causes ET insensitivity (Chang et al., 1993a). When the genomic clone of Arabidopsis *etr1-1* was transformed to tobacco, it caused ET insensitivity in the transgenic plants and strongly reduced basic pathogenesis-related gene expression and non-host resistance against *Pythium* spp. (Knoester et al., 1998). Here, we show that transformation of birch with the genomic copy of the Arabidopsis *etr1-1* mutant allele under its own promoter resulted in ET insensitivity in birch. Furthermore, the ET insensitivity was strong, which is demonstrated by the fact that MCP treatment of the transgenic trees did not decrease the ET-dependent O_3 lesion formation any further. Therefore, regulation of *ETR1* is most likely conserved among various plant species because apparently birch transcription factors recognized the Arabidopsis *ETR1* promoter and regulated its expression properly.

O_3 Lesions in ET-Insensitive Plants May Be Due to Defective HCN Removal

Insensitivity to ET provided only a partial protection against O_3, when the prevention of ET biosynthesis in the ET-insensitive transgenic birch blocked cell death completely. This suggests that ET modifies O_3-induced ET-dependent cell death downstream of ACS and both upstream and downstream of ET receptors. When ACC is oxidized to ET, stoichiometric amounts of ET and HCN are produced. Under nor-

mal circumstances, this HCN is efficiently detoxified by β-CAS. However, it has been suggested (Grossmann, 1996) that under specific conditions, strongly stimulated ET biosynthesis may result in necrotic cell death due to insufficient HCN detoxification. Infection of tobacco with tobacco mosaic virus caused elevated HCN and ACC levels, concomitant with a decrease in β-CAS activity (Siefert et al., 1995), and HCN accumulation was suggested to contribute to the cell death. Our results indicate that the birch *β-CAS* gene is ET-regulated. The *β-CAS* transcript accumulation was also considerably lower in response to O_3 when ET perception was disrupted. However, this was still accompanied by equal O_3-induced ET accumulation when compared with the wild-type birch, thus ACO was still producing HCN at an equal rate as ET. Hence, the HCN formed could be a likely candidate to mediate the O_3-induced cell death.

HCN may also relate to the ROS formation in the mitochondria: It has been shown that the HCN-resistant respiration decreased mitochondrial ROS formation in cultured tobacco cells via the alternative oxidase (AOX; Maxwell et al., 1999). It has also been shown with the Arabidopsis *etr1-1* mutant that AOX activation is ET dependent (Simons et al., 1999). The AOX gene is also induced by H_2O_2 (Robson and Vanlerberghe, 2002). Pellinen et al. (1999, 2002) have shown that O_3 induces H_2O_2 accumulation in birch. This suggests that AOX was most likely induced as well. Thus, attenuated induction of the HCN-resistant respiration together with defective HCN removal in ET-insensitive plants may result in inhibition of the normal mitochondrial respiration by HCN and thus cause increased ROS production in the mitochondria, which could be involved in the regulation of cell death. When the subcellular compartmentalization of O_3-induced H_2O_2 formation was studied in birch (Pellinen et al., 1999), increased ROS accumulation temporally coinciding with cell death was observed also in the mitochondria.

In this study, the defect in HCN detoxification may be considered an "artificial" effect caused by the strong ET insensitivity obtained with *etr1-1* transformation or MCP treatment. This strong ET insensitivity may be regarded as unlikely to be observed in wild-type plants. However, our results in Arabidopsis suggest that modulation of ET sensitivity takes place in O_3-exposed plants (H. Tuominen, K. Overmyer, M. Keinänen, and J. Kangasjärvi, unpublished data). Furthermore, it has been shown that in tomato, the genes encoding ET receptors are differentially induced by O_3 (Moeder et al., 2002) and during pathogen infection (Ciardi et al., 2000). The increased synthesis of "fresh," unoccupied receptors has been proposed to decrease ET sensitivity and to be involved in the desensitization of plants to ET when the ET responses need to be shut down (Bleecker and Kende, 2000; Ciardi and Klee, 2001). In tomato, the O_3-induced ET synthesis, ET-dependent H_2O_2 accumulation, and subsequent cell death were highly colocalized to the vicinity of the veins (Moeder et al., 2002). The ET-signaling-independent lesion formation in birch was also near the vascular system. Thus, it is completely possible that the simultaneous and extremely localized high ET biosynthesis in connection with decreased ET sensitivity caused by the induction of ET receptor genes can cause cell death through increased HCN formation and mitochondrial responses.

The deficient HCN removal in transgenic, ET-insensitive plants also has implications in other species that have been made insensitive to ET by transformation of mutant ET receptors; under conditions that cause highly elevated ET synthesis, sufficient HCN detoxification may not take place due to the ineffective induction of *β-CAS*.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Three birch (*Betula pendula* Roth) genotypes (V5818, K1898, and E9702) and ET-insensitive birch lines were propagated by in vitro tissue culture as described previously (Lemmetyinen et al., 1998). Copies of the clones (ramets) were planted and grown in peat:sand:vermiculite (6:1:1, v/v), fertilized with nitrogen:phosphorus:potassium (11:4:25) under greenhouse conditions, and used for experiments during either the 1st (3 month old) or 2nd year of their growth. Ramets were transferred into growth chambers with the photoperiod of 22 h of light/2 h of dark, light intensity of 300 μmol m^{-2} s^{-1} photosynthetically active radiation, temperature of 20°C/16°C (light/dark), and relative humidity of 50%/70% (light/dark) and allowed to acclimate to the chamber conditions at least for 4 d before the O_3, ET, and inhibitor treatments.

ET-insensitive birch were created by genetic transformation with a construct (pCGN1547) carrying the Arabidopsis ET receptor gene *ETR1* with the dominant *etr1-1* mutation with its own promoter (Chang et al., 1993a). Different birch clones (4212, E9678, E9702, JR1/4, K1659, K1898, V5818, and V5834) were chosen for *Agrobacterium* sp.-mediated (C58C1 pGV2260) transformation as described (Lemmetyinen et al., 1998). Clones V5818, K1898, and E9702 used in the other experiments reported here could not be successfully transformed (data not shown).

O_3, Chemical, and ET 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 an ozone analyzer (Dasibi 1008-RS, Amko Systems Inc., Ontario, Canada) as described by Pellinen et al. (1999). Wild-type birch clones (V5818, K1898, and E9702) were exposed to 200 nL L^{-1} O_3 for 8 h, and leaves were harvested at 0, 0.5, 1, 2, 4, 8, and 24 h. ET-insensitive birch ramets were exposed to 200 nL L^{-1} O_3 for 6 h, and leaves were harvested at 0, 6, 8, and 24 h. Wild-type birch ramets of the same clone were used as controls.

ET receptors were blocked in the wild-type clones (V5818 and K1898) and ET-insensitive transgenic birch line *BPetr1-1-35* with 1-MCP (EthylBloc, Laboratorium Van der Sprong bv, Netherlands). The treatments were carried out for 12 h in a sealed growth chamber with 300 nL L^{-1} MCP according to manufacturer's instructions, before or after the O_3 treatment. After each MCP pretreatment, the chamber was flow-through ventilated for 2 h before the O_3 exposure to avoid any possible chemical reactions between O_3 and MCP.

ET biosynthesis was blocked in the wild-type clone V5818 and transgenic birch line *BPetr1-1-35* with 1 mM or 1.5 mM AOA (Sigma-Aldrich) with 0.1% or 0.05% (v/v) Tween 20 (Fluka, Buchs, Switzerland), respectively, by spraying the abaxial side of the leaves. The control plants for AOA treat-

ment received 0.1% or 0.05% (v/v) Tween 20. Spraying was conducted 1.5 h before the onset of O_3 exposure and was repeated four times during the O_3 exposure.

ET treatment was conducted to test the ET sensitivity of the 3-month-old transgenic *etr1-1* clones by subjecting ramets to 50 μL L^{-1} ET for 3 d in a growth chamber.

ET, Ion Leakage, JA, and SA Determinations

The fully expanded leaves 1, 2, and 5 from the apex were pooled for ET evolution and ion leakage measurements from three ramets per clone per treatment. The ET and ion leakage determinations were carried out as described by Vahala et al. (2003).

The fully expanded leaves 3, 4, and 6 from the apex were used for JA and SA determinations at each time point. JA and SA were extracted and quantified with [1,2-^{13}C]JA and [^{13}C]SA as internal standards by gas chromatography-mass spectrometry as described by Baldwin et al. (1997), with the following modifications. The frozen tissues were ground in 2-mL microfuge tubes, spiked with an internal standard, extracted overnight with 1.5 mL of acetone:50 mM citric acid (7:3, v/v) at 4°C with vigorous shaking, and re-extracted for 15 min with 1 mL of the extraction solvent. After centrifugation (13,000 rpm for 8 min), the combined supernatants were divided in two aliquots (for free and conjugated compounds) and evaporated to an aqueous residue under vacuum at 30°C. For the analysis of conjugated SA, the sample was adjusted to 400 μL with deionized water, acidified with 100 μL of concentrated HCl (37%, w/v), and hydrolyzed for 1 h at 80°C. After hydrolysis, the aqueous solutions were extracted twice with 1 mL of diethyl ether and evaporated to dryness under vacuum at ambient temperature. The residues were redissolved in 1 mL of diethyl ether and loaded onto 100 mg of Supelclean LC-NH_2 SPE columns (Supelco, Bellefonte, PA). The columns were washed with 1.2 mL of chloroform:2-propanol (3:1, v/v), and the compounds were eluted with 1.5 mL of diethylether:formic acid (98:2, v/v). The eluates were evaporated to dryness under vacuum at ambient temperature, dissolved in 100 μL of diethyl ether, derivatized with ethereal diazomethane, and reconstituted in 40 μL of hexane.

The methyl esters of JA and SA were separated by gas chromatography (HP 6890, Agilent Technologies, Avondale, PA) on an Rtx-5MS column (30-m × 0.25-mm. i.d., 0.25-μm film thickness; Restek Corp., Bellefonte, PA) with a helium flow rate of 1 mL min^{-1} and injector temperature of 250°C, and detected by mass spectrometry (HP 5973) in a single-ion-monitoring mode (m/z 224 and 226 for JA, m/z 152 and 153 for SA). The temperature program was as follows, 1.5 min at 55°C, 10°C min^{-1} to 200°C, 20°C min^{-1} to 300°C, and hold at 300°C for 7 min.

cDNA Probes and Northern and Southern Analysis

Several degenerate oligonucleotide primers based on conserved amino acid domains were used to isolate cDNAs for the ET biosynthetic enzymes from birch. Reverse transcription of total RNA or poly(A^+) mRNA was conducted with the avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI), and the subsequent PCR was conducted with Dynazyme (Finnzymes, Espoo, Finland), *Taq*, or Pfu (Promega) DNA polymerases. To isolate birch ACS cDNA sequences, *OLE-4* and *OLE-5* upstream primers and *OLE-6* downstream primer were used as described (Botella et al., 1992).

Young leaf tissue collected from the apex of shoots was used for Southern analyses. Total DNA was extracted as described (Lodhi et al., 1994) and digested with *Kpn*I and *Eco*RI. For northern analysis, the fully expanded leaves 3, 4, and 6 from the apex were excised from three individuals per clone per treatment and frozen in liquid N_2 at each time point. Samples were stored at −70°C or −80°C until analyzed. The three independent samples were pooled, and total RNA was isolated as described (Chang et al., 1993b). Ten micrograms of total RNA was fractionated on 1% (w/v) formaldehyde agarose gels in MOPS buffer. RNA and DNA were capillary blotted overnight onto positively charged nylon membranes (Roche Diagnostics GmbH, Mannheim, Germany), and baked at 120°C for 30 min. [α-^{32}P]dCTP-labeled probes were prepared with High Prime random-priming labeling system (Roche Diagnostics) and purified on G-50 columns (Amersham Biosciences, Piscataway, NJ). Prehybridizations and hybridizations were carried out at 68°C in a solution containing 0.5 M $NaHPO_4$ and 7% (w/v) SDS. After hybridization, the membranes were washed under high-stringency conditions as described (Church and Gilbert, 1984). Hybridization signals were quantified with a phosphor imager and an image analysis program (Bas 1500, Fujifilm, Tokyo) and normalized against the 18S rDNA hybridization signal.

Photosynthesis Measurements

The net photosynthesis, stomatal conductance, and transpiration measurements were performed at 5 to 6 h after the onset of O_3 exposure with the LI-6400 photosynthesis system (LI-COR, Lincoln, NE). Measurements were performed on the third and fourth fully expanded leaves of three ramets per treatment. The mean value of two leaves measured from each individual ramet was calculated. All of the measurements were made under a 6400-02B red/blue LED light source at saturating light 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 30% to 40%.

Statistical Analysis and Quantification of Visual Damage

ANOVA was used to detect significant differences among clones and treatments. All data were checked for normality and heterogeneity of variances. Hormone concentrations were $\log_{10}(X)$ transformed and ion leakage arcsin transformed to meet the assumptions of ANOVA. Multiple comparisons of individual means and levels of factors were analyzed with a Tukey's HSD test, where appropriate. Correlations were analyzed with non-parametric Spearman's ρ test. Analyses were conducted with the SPSS v8.0 software package (SPSS Inc., Chicago). The O_3-induced visual damage was estimated as a percentage of the leaf area that was damaged 48 h after the onset of the O_3 exposure. All fully emerged leaves were counted. Two people conducted the quantification independently.

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