Plant Physiol. (1992) 99, 1321–1328
0032-0889/92/99/1321/08/$01.00/0

Received for publication November 21, 1991
Accepted February 26, 1992

Biochemical Plant Responses to Ozone[1]

III. Activation of the Defense-Related Proteins β-1,3-Glucanase and Chitinase in Tobacco Leaves

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ABSTRACT

A single pulse of O_3 (0.15 microliter per liter, 5 hours) induced β-1,3-glucanase and chitinase activities in O_3-sensitive and -tolerant tobacco (*Nicotiana tabacum* L.) cultivars. In the O_3-sensitive cultivar Bel W3, the response was rapid (maximum after 5 to 10 hours) and was far more pronounced for β-1,3-glucanase (40- to 75-fold) than for chitinase (4-fold). In the O_3-tolerant cultivar Bel B, β-1,3-glucanase was induced up to 30-fold and chitinase up to 3-fold under O_3 concentrations that did not lead to visible damage. Northern blot hybridization showed a marked increase in β-1,3-glucanase mRNA in cultivar Bel W3 between 3 and 24 hours following O_3 treatment, a transient induction in cultivar Bel B, and no change in control plants. The induction of β-1,3-glucanase and chitinase activities following O_3 treatment occurred within the leaf cells and was not found in the intercellular wash fluids. In addition, O_3 treatment increased the amount of the β-1,3-glucan callose, which accumulated predominantly around the necrotic spots in cultivar Bel W3. The results demonstrate that near-ambient O_3 levels can induce pathogenesis-related proteins and may thereby alter the disposition of plants toward pathogen attack.

PR[2] proteins account for marked quantitative changes in the soluble protein fraction of leaves infected with viruses or fungi (3, 7). Tobacco PR proteins have been classified into five groups and two of these groups contain proteins with β-1,3-endoglucanase (EC 3.2.1.39) and endochitinase (EC 3.2.1.14) activity (3). Most of the TMV-induced proteins were shown to have an acidic nature and to be localized extracellularly. Basic isoforms have also been characterized and were found to accumulate in vacuoles (3, 7). When β-1,3-glucanase and chitinase are applied in combination, they inhibit *in vitro* the growth of fungi that contain β-1,3-glucans or chitin as cell wall polymers. As a consequence, a role for these glucanohydrolases was postulated in disposing plants for defense against microbial infection (4, 7). Both proteins were also detected in flowers, older leaves, and roots of noninfected plants, which may reflect a constitutive defense system but also points to a possible endogenous function in plant development (9, 12, 27).

β-1,3-Glucanase and chitinase activities are also induced by hormonal and chemical treatments (4, 7). When anthropogenic chemicals of environmental significance are considered, ethylene as well as heavy metals represent possible inducers of PR proteins in vegetation. In addition to being of plant origin, ethylene is an air pollutant in industrialized areas (29). Ethylene emissions from unburned gas and industrial processes can be toxic to nearby vegetation (29). Heavy metal salts, *e.g.* $HgCl_2$ and $NiCl_2$, were also shown to induce PR proteins in plants (3, 4). However, heavy metal concentrations similar to those used under experimental conditions are found only in strongly contaminated soils.

In contrast with the restricted environmental occurrence of high ethylene and heavy metal salt concentrations, ambient levels of O_3, the most important photooxidant in Europe and North America, are close to the range that influences plant performance, including biochemical and physiological changes, accelerated senescence of organs, and reduction in crop yield (14, 24, 29).

We recently found that exposure to O_3 changes the protein pattern in leaves of tobacco (*Nicotiana tabacum* L.) (17) and needles of *Picea abies* (L.) Karst. (25). Several O_3-induced proteins were found but their nature was not elucidated in these studies. We now provide evidence that near-ambient O_3 concentrations significantly increase the activities of two defense-related proteins, β-1,3-glucanase and chitinase, in O_3-sensitive (Bel W3) and -tolerant (Bel B) tobacco cultivars (18). Even though low levels of enzyme activities were detected in the IWF of control plants, their induction by O_3 was only observed intracellularly. Furthermore, we show for the first time that exposure to a pollutant gas affects plant protein induction at the mRNA level.

MATERIALS AND METHODS

Plant Material and Conditions of Treatment

Tobacco (*Nicotiana tabacum* L. cv Bel W3 and Bel B) plants were grown under a 16 h/8 h light/dark regimen in pollutant-free air as described previously (18). Experiments were conducted with 8- to 10-week-old plants. They were exposed to a single pulse of O_3 (standard fumigation, 0.15 μL/L) for 5 h

[1] Supported by grants from Bundesministerium für Forschung und Technologie, Deutsche Forschungsgemeinschaft, and from Fonds der Chemischen Industrie.

[2] Abbreviations: PR, pathogenesis-related; TMV, tobacco mosaic virus; IWF, intercellular wash fluid; SSC, 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0.

in Plexiglas chambers placed in a walk-in growth cabinet. Outdoor O_3 concentration data of July 1990 at Schönenbuch, Switzerland, were provided by Dr. S. Leonardi (Institut für Angewandte Pflanzenbiologie, Schönenbuch) and were simulated as 2-h means by a computer-controlled system for O_3 dosage and analysis. O_3 was generated by electrical discharge in dry oxygen. O_3 concentration was measured with a CSI 3100 analyzer (Messer-Griesheim, München), which was periodically calibrated as described (18). Control plants were cultivated in pollutant-free air in parallel chambers. Leaf injury was estimated according to Langebartels *et al.* (18). Stomatal conductance was measured under the chamber conditions with portable gas-exchange equipment (LCA-2 Analyzer, Parkinson Leaf Chamber, air supply by mass flow meter; ADC, GB-Hoddesdon).

Tissue Sampling and Preparation of Intercellular Wash Fluid

Leaf discs (1 cm diameter, 0.2 g) were cut with a cork borer from the midleaf of leaves 3 and 4 avoiding major veins. The first leaf of >8 cm length from the shoot apex was counted as leaf 1. Discs were rapidly frozen in liquid nitrogen and stored at −80°C. IWF was prepared from whole leaves or leaf halves that were vacuum-infiltrated with 62 mM sodium acetate buffer (pH 5.5) or 50 mM Tris-HCl (pH 7.0), 100 mM KCl (18). The IWF was collected by centrifuging the rolled leaves at 1000*g* for 10 min. This method led to less than 0.5% contamination with intracellular markers (18). Similar IWF yields and enzyme activities were obtained when leaf stems and major veins were sealed with wax before centrifugation.

Assays for β-1,3-Glucanase and Chitinase Activities

Leaf material (0.2 g) was ground in liquid N_2 in a mortar and pestle. The powder was suspended in 1 mL of 62 mM sodium acetate buffer (pH 5.5). The homogenate was centrifuged at 15,000*g* for 10 min (4°C), and the clear supernatant was used for enzyme measurements. β-1,3-Glucanase activity was assayed fluorimetrically with laminarin as substrate (adapted from ref. 16). Plant extract (50 μL, diluted up to 1:10 with sodium acetate buffer) was mixed with 0.1 mg laminarin in 0.1 mL of 50 mM sodium phosphate buffer (pH 6.0) and incubated 30 min with shaking at 37°C. The reaction was stopped by adding 50 μL of 4 N NaOH and placing the samples in a water bath at 80°C for 5 min. Aniline blue (0.4 mL, 0.1% [w/v], Serva), 1 N HCl (0.2 mL), and 1 M glycine-NaOH buffer (pH 9.5, 0.6 mL) was added, and the mixture incubated for 20 min at 50°C and overnight at room temperature. A stable sirofluor-laminarin complex was formed within 1 h. The samples were measured in a fluorimeter (F-3000, Hitachi) at 480 nm (excitation 400 nm). All assays were performed in triplicate. The test was linear between 0.005 and 0.15 mg laminarin/assay. Enzyme activity is expressed in katal/g fresh weight based on D-glucose reducing equivalents from laminarin (8).

Chitinase activity was assayed radiometrically according to Boller *et al.* (5). The assay mixture contained 0.1 mL of plant extract, 50 μL of 0.1 M sodium phosphate buffer (pH 6.5), and 0.1 mL of regenerated [^{3}H]chitin (1.6 kBq, 18.3 kBq/mg). The mixture was incubated at 37°C for 10 min, and the reaction stopped with 0.25 mL of 1 M TCA. After centrifugation (12,000*g*, 10 min), 0.25 mL of the supernatant was carefully removed and its radioactivity determined. All assays were run in triplicate.

Representative results are shown from at least two experiments with three replicate measurements. When indicated, the LSD multiple range test was used to test for differences among treatment means (Statgraphics, STSC Inc., Rockville, MD).

Determination of Callose

Callose content of the leaves was determined according to Köhle *et al.* (16). For histochemical staining, leaves were immersed in 70% ethanol for 30 s and were stained with 0.1% decolorized aniline blue (pH 8.5). An Orthoplan fluorescence microscope (Leitz, Wetzlar) with Leitz filter sets A (UV) and I 2/3 (blue) was used.

RNA Isolation and Northern Blot Analysis

Frozen leaf material was ground in a mortar under liquid nitrogen cooling. The fine powder was allowed to thaw in 1 volume of extraction buffer (50 mM Tris-HCl buffer [pH 8.0] containing 4% [w/v] sodium *p*-aminosalicylate [Sigma] and 1% [w/v] sodium-1,5-naphthalene disulfonate [Kodak]). The homogenate was extracted twice with 1.6 volumes of phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v). RNA was precipitated from the final aqueous phase in 1 M LiCl, 2 M urea, 1 mM EDTA at 4°C overnight. The precipitate was collected by centrifugation and resuspended in 40 mM Tris-HCl (pH 7.5) containing 30 mM sodium acetate, 5 mM EDTA, and 1% (w/v) SDS. RNA was precipitated by the addition of 3 volumes of ethanol at room temperature. The precipitate was washed twice with 75% ethanol containing 10 mM Tris-HCl, 1 mM EDTA, and 0.3 M sodium acetate. RNA concentration was determined by absorption measurement at 260 nm. The yield of total RNA was approximately 240 μg/g fresh weight. The method for northern blot analysis was that of Maniatis *et al.* (19). Total RNA was denaturated and electrophoresed on horizontal 1.2% agarose formaldehyde gels (15 μg/lane). The *Pst*I insert of cDNA of tobacco β-1,3-glucanase (pGL 43, [21]) and the *Hin*dIII insert of cDNA of soybean actin (pSAc 3, [26]) were used for hybridization. The DNA probes were labeled with [α-^{32}P]dCTP using a nick translation kit (Amersham). Pre-hybridization and hybridization was carried out at 42°C in 50% formamide containing 5 × SSC, 5 × Denhardt's solution, and 1% SDS (19). Filters were washed at 65°C with 0.1 × SSC, 0.1% SDS (glucanase DNA probe) or with 1 × SSC, O.1% SDS (actin DNA probe). Autoradiograms (Amersham Hyperfilm MP) were scanned with a densitometer (Ultrascan XL, Beckman-Broma).

RESULTS

O_3 Induction of β-1,3-Glucanase and Chitinase Activities

Enzyme activities in leaves of the tobacco cultivars Bel W3 (O_3- sensitive) and Bel B (O_3-tolerant) were measured before and after a 5-h exposure to 0.15 μL/L O_3, as well as on

further cultivation in pollutant-free air. This treatment resulted in foliar lesions on approximately 30% of the leaf area of middle-aged leaves (position 3 and 4 from the top) in cv Bel W3. The lesions developed between 15 and 48 h after the onset of exposure. No visible injury was apparent on leaves of the O_3-tolerant cv Bel B (18).

Exposure to O_3 increased the β-1,3-glucanase activity of the O_3-sensitive cv Bel W3 up to 40-fold relative to control plants that were kept in pollutant-free air (Fig. 1a). The response was rapid and near maximum values were reached after 10 h. The O_3-tolerant cv Bel B showed a minor induction, which was approximately 10-fold. Chitinase activity increased already during the 5-h exposure period (Fig. 1b). Both cultivars showed comparable induction factors (three- to fourfold). Activities of both enzymes remained at elevated levels for at least 2 d in the O_3-sensitive cultivar, whereas they returned to control activities after 24 h in cv Bel B. The basal levels of β-1,3-glucanase and chitinase were similar in both cultivars and did not change during exposure to pollutant-free air.

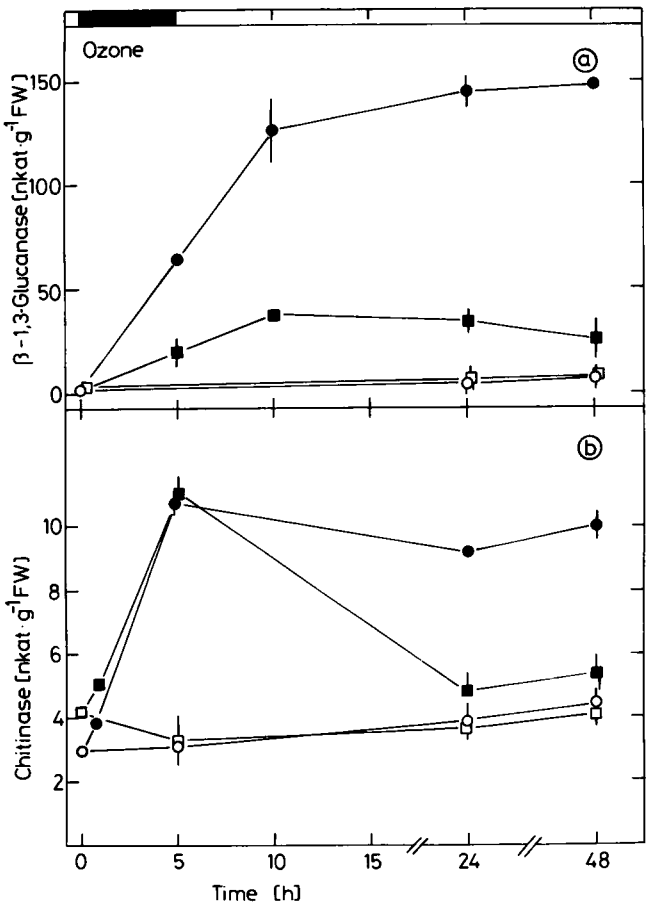

Figure 1. Time course of β-1,3-glucanase (a) and chitinase (b) induction by O_3. Tobacco plants (10-week-old) were exposed to 0.15 μL/L O_3 for 5 h or were held in pollutant-free air. Both groups of plants were further cultivated in pollutant-free air for the times indicated. Closed circles and squares, O_3-treated plants of cv Bel W3 and Bel B, respectively; open circles and squares, control plants of cv Bel W3 and Bel B. Bars represent ± SE (n = 4).

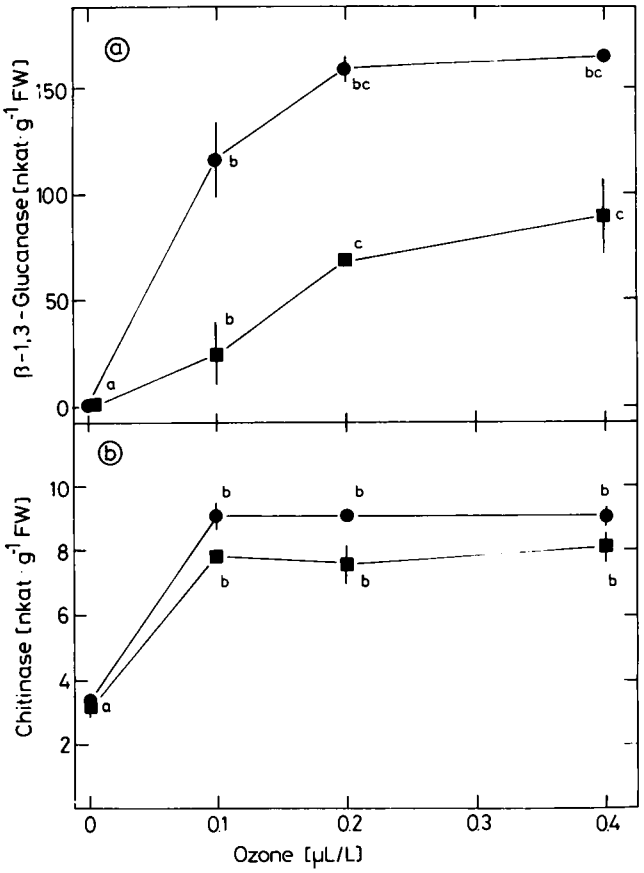

Figure 2. Effect of O_3 concentration on β-1,3-glucanase (a) and chitinase (b) induction. Tobacco plants were held in O_3-containing air for 5 h and in pollutant-free air for further 24 h. Enzyme activities were measured after 24 h as described in "Materials and Methods." Circles, cv Bel W3; squares, cv Bel B. Means (n = 4) followed by the same letter are not significantly different ($P < 0.05$) according to the LSD multiple range test. Bars represent ± SE.

Dose-response studies showed that β-1,3-glucanase activity was induced up to 70-fold at 0.2 μL/L O_3 (5 h) in leaves of cv Bel W3 (Fig. 2a). A significant induction with near maximum levels was also found at 0.1 μL/L O_3, which is close to ambient summer O_3 concentrations. Bel B plants showed a nearly linear O_3-dependent increase of β-1,3-glucanase activity up to 0.4 μL/L O_3, which is the threshold concentration for visible injury (18). Chitinase activity was maximally induced (threefold) in both cultivars at the lowest O_3 dose applied (0.1 μL/L, 5 h) (Fig. 2b).

To examine the induction potential of ambient pollutant profiles, O_3 data as well as climatic parameters for two days in July 1990 at Schönenbuch, Switzerland, were simulated as 2-h means in closed chambers. The 24-h means were 0.05 and 0.065 μL/L O_3 and the maximum concentrations were 0.09 and 0.1 μL/L O_3 for the 12th and 13th of July 1990, respectively. The 2-d treatment resulted in 19% injury on leaves of cv Bel W3 and a β-1,3-glucanase activity of 114 ± 8 nkat/g fresh weight, which was significantly higher ($P < 0.01$) than in control plants (21 ± 7 nkat/g fresh weight).

Effects of O_3 on Plant Distribution and Compartmentation of Enzyme Activities

Leaves of tobacco cv Bel W3 were most sensitive to O_3 during later stages of leaf enlargement (maximum 30% injury in leaves 3 and 4 from the top of the plant) (Fig. 3a). Tobacco Bel B was not visibly injured by the standard exposure conditions used (0.15 μL/L O_3, 5 h). The stomatal conductance was similar in both cultivars, was highest in leaves 3 and 4 (38 ± 9 mmol m^{-2} s^{-1}), and decreased to 18 ± 5 mmol m^{-2} s^{-1} in leaf 7. The control levels of β-1,3-glucanase activity were near the detection limit in young and middle-aged leaves and were highest in leaves 6 and 7 of both cultivars (Fig. 3b). O_3 markedly increased the β-1,3-glucanase activity in all leaves assayed. Activities were always higher in cv Bel W3 than in Bel B. Maximum enzyme induction was observed in leaves 3 through 5 (approximately 120 nkat g^{-1} fresh weight). The enzyme activities of the older leaves 6 and 7 were induced by only 50 to 100% over already high endogenous levels. Chitinase activity was comparable in all leaves of control plants of both cultivars and increased up to threefold by the pollutant treatment (data not shown).

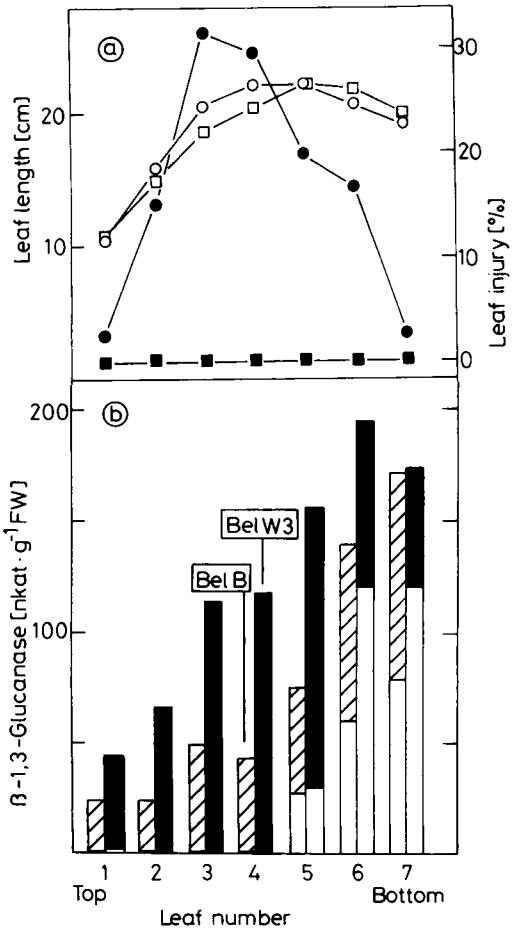

Figure 3. O_3-induced lesion formation and β-1,3-glucanase activity in Bel W3 and Bel B tobacco leaves. Plants (10-week-old) were exposed to 0.15 μL/L O_3 for 5 h or to pollutant-free air. a, Length of individual leaves and percentage of leaf injury. Closed circles and squares, leaf injury of cv Bel W3 and cv Bel B, respectively; open circles and squares, leaf length of cv Bel W3 and cv Bel B. b, β-1,3-Glucanase activity in control leaves (open columns) and O_3-exposed leaves (hatched columns, Bel B; filled columns, Bel W3). Means from three replicate plants. Leaves are counted from the top of the plant.

To determine whether β-1,3-glucanase and chitinase activities were localized intra- or extracellularly, we compared the activities in the IWF obtained by vaccuum infiltration of the leaves and in the residual leaf material. Phloem and xylem sap contamination of the IWF was excluded by sealing the cuttings of leaf stems and major veins with wax before centrifugation. The β-1,3-glucanase and chitinase activities of O_3-treated plants (0.15 μL/L, 5 h) and the induction factors compared with zero time levels are shown in Table I. Before the start of exposure to O_3, 30 to 50% of the basal β-1,3-glucanase activity was detectable in the IWF. The induction (77-fold in cv Bel W3 and 34-fold in Bel B), however, occurred only intracellularly in both cultivars. β-1,3-Glucanase activity in the IWF increased twofold in cv Bel W3, and was even reduced in cv Bel B. Chitinase activity was predominantly (95%) associated with the intracellular compartment in control plants (Table I). Its compartmentation was not changed by the O_3 treatment, which resulted in a 1.5- (Bel B) to 1.9-fold (Bel W3) increase in chitinase activity of the residual leaf and a lower increase in the IWF.

Callose Formation in O_3-Treated Plants

The callose content of leaves from control plants, as determined by aniline blue staining (16) in leaf extracts, was comparable in both cultivars (0.3 ± 0.02 mg laminarin equivalents/g fresh weight). An increase by a factor of two (Bel B) or three (Bel W3) was observed 24 h after the start of the standard 5-h O_3 exposure. O_3-induced callose formation was also analyzed by fluorescence microscopy and histochemistry. Figure 4a shows the appearance of the typical spot-like bifacial lesions on middle-aged leaves of cv Bel W3 after 48 h. When lower leaf surfaces were examined under UV light (excitation maximum 340–380 nm, barrier filter 430 nm), distinct regions, comprising some 10 to 100 cells, showed a highly intense blue autofluorescence after 8 h (Fig. 4b), whereas the other tissue exhibited the usual red Chl fluorescence (upper part in Fig. 4b). The blue fluorescing regions, which presumably indicate an altered polyphenol composition of the cells (*cf.* ref. 22) were the first visible sign of lesion formation and later developed into necrotic spots. Yellow/green fluorescence due to aniline blue was detected after 48 h within the necrotic area and particularly in the living cells surrounding the lesions (Fig. 4c). Under blue light (450–490 nm, 525 nm) a sharp boundary layer between yellow fluorescing necrotic areas and healthy tissue was visible (Fig. 4d).

O_3 Induction of β-1,3-Glucanase mRNA

The leaves of both tobacco cultivars were assayed for their β-1,3-glucanase mRNA content by northern blot analysis. A cDNA probe for a basic isoform of β-1,3-glucanase from tobacco (pGL43) (21) was used, which hybridized with a

Table I. *Time Course of β-1,3-Glucanase and Chitinase Activities in Intercellular Wash Fluid and Leaves of O_3-Treated Tobacco Plants*

Plants were treated with O_3 (0.15 μL/L, 5 h) and the middle-aged leaves were harvested at the times indicated. The enzyme activity in the IWF and in the residual leaf is expressed in nkat/g of intact leaf (activity) or as factor over zero time controls (Factor). Means are given ± SE for three replicate plants. n.d., Not determined.

	Time	Bel W3				Bel B			
		IWF		Leaf		IWF		Leaf	
		Activity	Factor	Activity	Factor	Activity	Factor	Activity	Factor
β-1,3-Glucanase	0 h	0.5 ± 0.01	1	1.5 ± 0.2	1	0.7 ± 0.2	1	1.5 ± 0.4	1
	16 h	0.5 ± 0.03	1.1	40.5 ± 3.4	33	0.6 ± 0.1	0.8	41.2 ± 5.6	34
	48 h	1.0 ± 0.04	2.0	91.9 ± 6.2	77	0.3 ± 0.04	0.4	11.5 ± 6.2	9
	72 h	0.3	0.5	19.6	16	0.15	0.2	17.0 ± 6.1	14
Chitinase	0 h	0.24 ± 0.02	1	3.60 ± 0.28	1	0.29 ± 0.05	1	3.42 ± 0.2	1
	16 h	0.25 ± 0.03	1.0	5.33 ± 0.05	1.5	0.30 ± 0.05	1.0	5.03 ± 0.05	1.5
	24 h	0.36 ± 0.07	1.5	5.26 ± 0.79	1.5	0.30 ± 0.05	1.0	3.99 ± 0.18	1.2
	48 h	0.25 ± 0.03	1.0	6.90 ± 0.40	1.9	0.23 ± 0.03	0.8	3.79 ± 0.11	1.1
	72 h	0.22 ± 0.01	1.0	5.65 ± 0.08	1.6	n.d.		4.47 ± 0.04	1.3

Figure 4. Analysis of O_3-induced lesions in tobacco Bel W3. a, Necrotic spots 48 h postexposure (0.15 μL/L O_3, 5 h). b-d, Histochemical staining of O_3-exposed leaves with decolorized aniline blue (lower leaf surface). b, Blue autofluorescence of leaf cells 8 h after the start of exposure, UV light excitation (excitation at 340–380 nm, emission at 430 nm). c, Aniline blue fluorescence of the necrotic area after 48 h, UV light excitation. d, Necrotic lesion after 48 h, blue light excitation (excitation at 450–490 nm, emission at 515 nm). Bars, 100 μm (b-d).

single RNA species corresponding in size to the approximately 1.6-kilobase β-1,3-glucanase mRNA found in cultured tobacco tissue (21). Based on intensity of labeling, β-1,3-glucanase mRNA of cv Bel W3 was induced approximately threefold after 3 h of treatment (Fig. 5). The mRNA levels further increased after the end of the exposure period and were approximately 15-fold after 24 h. The β-1,3-glucanase mRNA was transiently induced (maximum at 5 h) in cv Bel B. Control plants of cv Bel W3 showed a weak increase in mRNA after 1 h, which may be due to the transfer of the plants to the exposure cuvettes (Fig. 5). When the filters were rehybridized with a constitutively expressed actin cDNA clone (pSAc3 actin) (26), the mRNA contents did not change with treatments, indicating that there was no nonspecific shift in the relative amounts of rRNA and mRNA (data not shown).

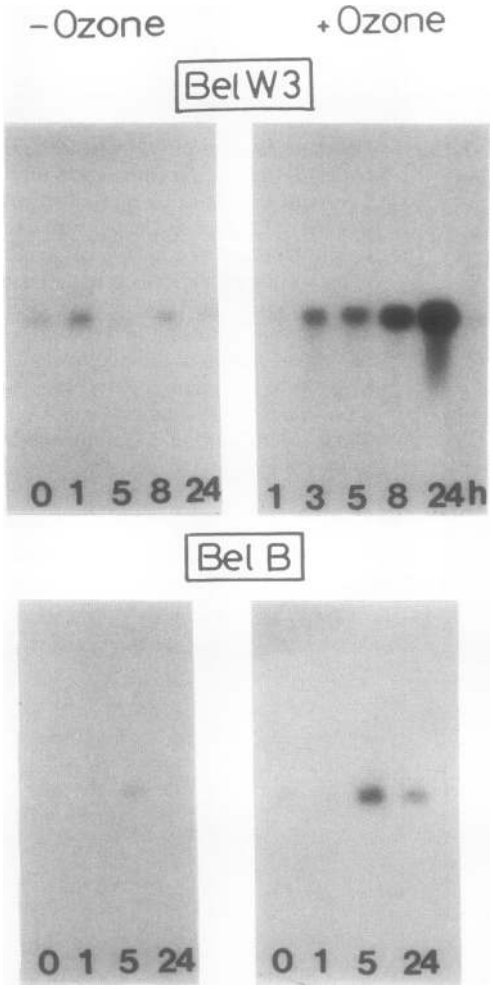

Figure 5. Northern blot analysis of total RNA isolated from O_3-exposed and control plants of tobacco Bel W3 (O_3-sensitive) and Bel B (O_3-tolerant). Plants were treated with O_3 (0.15 μL/L) for 5 h and were postcultivated without pollutant for the times indicated. Control plants (minus O_3) received pollutant-free air. Fifteen micrograms of of total RNA were loaded in each lane of 1.2% agarose gels containing formaldehyde.

DISCUSSION

Induction of Enzyme Activities and β-1,3-Glucanase mRNA by O_3

Treatment of tobacco plants with O_3 increased the activities of β-1,3-glucanase and chitinase, which are two components of the inducible response of plants to pathogens (7). The time course of induction and the dose-dependence showed that the response was rapid (maximum 5–10 h after the start of exposure), massive (up to 4-fold for chitinase and up to 70-fold for β-1,3-glucanase), and persisted up to 48 h in the O_3-sensitive cv Bel W3. The increase in β-1,3-glucanase activity was accompanied by a parallel induction of mRNA for basic β-1,3-glucanase. This induction already occurred after 3 h of exposure. Accumulation of endohydrolases is thus among the most rapid responses of plants toward O_3. The time course resembles that of arginine decarboxylase activity, a biosynthetic enzyme for the diamine putrescine, in tobacco Bel B (18). The time course of O_3 activation of β-1,3-glucanase and chitinase was similar to that described for exposure of tobacco plants to ethylene (4), and was faster than that following TMV infection (30) or changes in auxin and cytokinins in cultured cells (21). The induction occurred markedly before the onset of local lesion formation (between 15 and 48 h) in cv Bel W3 and was independent of visible damage in cv Bel B.

The level of β-1,3-glucanase mRNA and the activities of both enzymes decreased in the O_3-tolerant cv Bel B when O_3 was withdrawn. On the other hand, the response of cv Bel W3 was detected up to 48 h, which indicates that a persistent stimulus was present. Enzyme activities of this cultivar correlated with the degree of visible symptoms. A prolonged accumulation of endohydrolases therefore seems to be coupled to lesion formation, a phenomenon also found in TMV-infected tobacco plants (30). The induction factors for β-1,3-glucanase activity varied from 20- to 70-fold in independent experiments, but were only 2- to 4-fold for chitinase. The high induction of β-1,3-glucanase activity correlated with a marked increase in β-1,3-glucanase mRNA content (Fig. 5), and a weak induction of chitinase was also observed at the mRNA level when a cDNA clone for a basic chitinase (27) was used for hybridization (D. Ernst, M. Schraudner, C. Langebartels, unpublished data). The difference in induction for these enzymes is in contrast with that seen in studies with ethylene- or pathogen-treated plants, which often show a closely coordinated response (4). On the other hand, ethylene treatment of bean predominately induced chitinase, and to a lesser extent β-1,3-glucanase activity (5). The strong activation of β-1,3-glucanase further correlated with an accumulation of its putative substrate, the β-1,3-glucan callose (*cf.* ref. 1). The callose content of the leaves was induced two- (Bel B) to threefold (Bel W3). Callose was present within the necrotic areas and particularly in the living cells around the lesions in the O_3-sensitive cv Bel W3 (Fig. 4). Cell wall callose deposition is also known as a rapid event following pathogen attack or elicitor treatment of plant cells (7, 16). Callose formation and β-1,3-glucanase activation may both be part of the inducible plant defense system toward pathogens (4, 7). β-1,3-Glucanase and other hydrolases may, however, also be involved in the use of monomers from polysaccharides of

necrotized areas. This function may occur in parallel to pathogen defense, but more generally might be part of a developmental process to recover useful products from discarded leaf areas or whole organs.

The responses in our tobacco system seem to be saturated with 5-h pulses of 0.1 or 0.2 μL/L O_3 for chitinase and β-1,3-glucanase, respectively. These O_3 levels are close to summer concentrations found in Europe and North America (peak values between 0.1 and 0.25 μL/L) (29). To test the effect of ambient O_3, tobacco Bel W3 plants were exposed to simulated O_3 values of two representative days in July 1990. This treatment resulted in a fivefold increase in β-1,3-glucanase activity above control levels. As the O_3 concentrations show an annual time course with highest values in summer, the control levels of endohydrolases in test plants may differ considerably depending on the respective outdoor or greenhouse O_3 concentrations. Pollutant concentrations therefore have to be controlled carefully in any study on plant-pathogen interactions because the disposition state of plants toward biotic stresses (11) may be altered by ambient O_3 levels.

Tissue and Cell-Specific Localization

β-1,3-Glucanase activities in plants cultivated in pollutant-free air were near the detection limit in young and middle-aged leaves, but were markedly higher in older leaves (Fig. 3). A similar pattern has been observed for β-1,3-glucanase and chitinase in other tobacco cultivars (9, 12, 27). O_3 induction occurred predominantly in the middle-aged leaves 3 to 5, which were also visibly damaged in cv Bel W3. These leaves had the highest stomatal conductance and were in that stage of leaf extension during which intercellular air spaces are formed. Fully functional stomata and intercellular spaces allow a maximum of diffusion of gases throughout the leaf. Thus, the O_3 concentration present in the leaf interior most probably determined the extent of β-1,3-glucanase induction. This result can be generalized for all externally applied gases, including ethylene.

The distribution of β-1,3-glucanase and chitinase activities between internal and external cell space was determined following vacuum infiltration of O_3-treated leaves. Some 30 to 50% of the control β-1,3-glucanase activity was found in the IWF of leaves in both cultivars. Chitinase activity of control plants, on the other hand, was predominantly (>95%) detected within the cells. Similar results were obtained with ethylene-treated bean plants in which minor amounts of β-1,3-glucanase, but not chitinase, were present in the IWF (20). O_3 treatment induced primarily the intracellular isoforms of the enzymes in our tobacco system. Intra- and extracellular isoforms of β-1,3-glucanase and chitinase are known to be differentially induced by several types of stress (7, 10). The ethylene-inducible isoforms of bean and tobacco plants are predominantly or exclusively located in the vacuole (15, 20). Because the induction of both enzyme activities in our tobacco system mainly occurred intracellularly and because mRNA hybridized to a basic β-1,3-glucanase clone was stimulated, vacuolar isoforms are probably also induced by O_3 as they are in ethylene-treated plants.

Ethylene or Reactive Oxygen Species as Mediators of O_3 Effects

An enhanced release of ethylene is often associated with the response of plants to microbial or chemical stress (13). Furthermore, exogenously applied ethylene induces β-1,3-glucanase and chitinase activities in various plant species (1, 4). A high level of ethylene formation, however, is not always required for an induction of endohydrolases (3, 4). Ethylene is well known to be induced by an exposure of plants to O_3 (29) and may represent a possible mediator of O_3 effects on β-1,3-glucanase and chitinase. In the tobacco cv Bel W3 studied here, O_3 induces a surge of ethylene emission that reaches a peak, after 1 to 2 h, before declining to the original levels after 5 h (18). The emission of ethylene also correlates with the degree of induction of β-1,3-glucanase activity (18, Fig. 2). In the tolerant cv Bel B, however, the induction of enzyme activity and mRNA contents were found at O_3 levels where no significant changes in ethylene emissions, 1-aminocyclopropane-1-carboxylic acid, or malonyl-1-aminocyclopropane-1-carboxylic acid contents occurred (18), suggesting that other, more direct mechanisms exist to mediate the observed O_3 effects.

There is increasing evidence that defense reactions in plants can be mediated by perturbations in the highly regulated oxidative state of the apoplast (10). A burst of activated oxygen species occurs extracytoplasmatically in plant tissues in response to pathogen attack and elicitor treatment (2, 10). H_2O_2 applied exogenously leads to phytoalexin formation in soybean cells (2). O_3 in the leaf interior would rapidly react with components of the cell wall and the apoplastic fluid and can be transformed to singlet oxygen, H_2O_2, hydroxyl radicals, and superoxide anion radicals (14). O_3 and O_3-derived oxyradicals may be scavenged by low mol wt constituents of the apoplast such as ascorbic acid (14) and polyamine conjugates (6, 18). Concentrations of activated oxygen species that exceed the antioxidative capacity of the apoplast may be part of the signal chain leading to defense-gene activation (*cf.* ref. 10). This general hypothesis is supported by the recent findings of regulatory gene regions in bacteria (28) and animal cells (23) that respond to oxidative stress or to antioxidants. Storz *et al.* (28) have shown that an inactive reduced OxyR protein factor is constitutively present in bacteria. This protein is rapidly transformed to the active oxidized form by low amounts of H_2O_2 and initiates transcription of oxidative stress-inducible genes *in vitro*. It is tempting to speculate that molecules that are activated by an oxidative process also exist in plant cells. Activation may occur either by oxygen species produced by the plant in response to pathogen attack or by O_3-derived species as described here. The extracellular activated oxygen species may then trigger endogenous oxygen radical production in the cell so that effects also arise on organelles, *e.g.* in chloroplasts and in the nucleus. These common mechanisms may be the key to the similarity of the plant's reactions to both types of stress.

ACKNOWLEDGMENTS

The authors thank Dr. F. Meins (Friedrich Miescher-Institut) and Dr. R.B. Meagher (University of Georgia) for the kind gift of the β-1,3-glucanase and actin clones, respectively. We wish to acknowledge

the continuous support for the project provided by Prof. Dr. Dr. h.c. H. Ziegler (Technische Universitút München) and thank Dr. E. Back (BUWAL, Bern) for help in the initial stages of the study, and Dr. D. Inzé (Rijksuniversiteit Gent) for critically reading the manuscript. We also like to thank Lucia Gössl, Andreas Steiner, Christian Zwiener, and Peter Bader for expert technical assistance, and our colleagues for their comments and criticism.

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