

Ozone-induced changes of β -1,3-glucanase, chitinase and 'pathogenesis-related' protein 1b in tobacco plants

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Abstract

Treatment of the ozone-sensitive tobacco cultivar Bel W3 with an ozone pulse (0.15 μ l/l, 5 h) markedly increased the mRNA level of basic β -1,3-glucanase and to a lower degree that of basic chitinase. The increase of β -1,3-glucanase mRNA level occurred within 1 h and showed a transient maximum. Seventeen hours after ozone treatment, the β -1,3-glucanase mRNA level decreased to lower values. The increase of basic chitinase mRNA level was delayed and was less pronounced than that of β -1,3-glucanase mRNA. Cultivar Bel B showed only a small increase of β -1,3-glucanase mRNA level after the same ozone treatment, whereas its basic chitinase mRNA was more strongly induced. Prolonged ozone treatment for 2 days of tobacco Bel W3 led to a persistent level of β -1,3-glucanase and basic chitinase mRNAs, as well as to an increase of acidic chitinase and 'pathogenesis-related' (PR) 1b mRNA levels. The results indicate that genes so far considered to code for PR proteins may also be involved in the plant response to oxidative stress.

Introduction

Ambient ozone concentrations in Europe and North America frequently reach levels (0.1–0.2 μ l/l) known to be toxic to plants [19, 45]. Controlled fumigation experiments with ozone revealed that low levels (<0.15 μ l/l) affect plant growth and development [19, 45]. Leaf damage results in bleaching, lesions, chlorosis and necrosis of plants [45]. Exposure to subacute ozone levels can result in the induction of biochemical and physiological processes in the plant. These reactions are part of the plant's defence against oxidative stress [12, 19, 22, 34, 36, 38]. The plant hormone ethylene seems to be involved in various

of the ozone-induced reactions [25, 29]. The hypersensitive tobacco line Bel W3 has been used for many years as a biomonitor for ozone [17, 21]. Large amounts of ethylene are produced by the ozone-sensitive tobacco cultivar Bel W3 after a short ozone exposure, whereas the tolerant cultivar Bel B shows no significant induction [25]. Ethylene is known as a plant stress hormone [48] and treatment of plants with ethylene results in an increased synthesis of hydrolases such as β -1,3-glucanases and chitinases [4, 16, 23, 26, 48]. In many plants chitinase and glucanase also accumulate upon pathogen attack or elicitor treatment [3, 11, 20, 26, 33].

Although the induction of β -1,3-glucanase and

chitinase has been studied at the molecular level [6, 20, 31, 33, 47, 49], little is known about the effects of environmental factors such as air pollutants on these genes. In a previous paper we reported an increase of β -1,3-glucanase and chitinase activities in ozone-treated tobacco [39]. The present study extends these findings with a different expression pattern of PR genes in the two tobacco cultivars analysed and indicates that genes for acidic and basic PR proteins respond differently to ozone.

Materials and methods

Growth conditions

Ozone-sensitive (*Nicotiana tabacum* (L.) cv. Bel W3) and ozone-tolerant (cv. Bel B) tobacco plants were cultivated for 12 weeks under a 12 h day/night cycle in an environmental growth chamber. Light conditions (15 000 lx), temperature (day 25 °C, night 20 °C) and humidity (75%) were as previously described [25].

Fumigations

Fumigations were performed according to Langebartels *et al.* [25]. Briefly, plants were kept in exposure chambers and were treated with either ozone or clean air. Pollutant-free air was obtained by passing ambient air through a filter system. Ozone was generated by electrical discharge in dry oxygen. The desired ozone concentration (0.15 μ l/l, standard condition) was obtained by computer control. Light, temperature and humidity conditions were the same as in the environmental growth chambers.

Determination of leaf injury

Leaf injury as a percentage of total leaf area was scored on leaves 3 and 4, taking the youngest leaf greater than 8 cm in length as number 1 [17, 25]. All analyses were carried out with leaves 3 and 4,

as these leaves were most sensitive to ozone [25, 39].

Harvesting plant tissue

Five hours after the onset of ozone treatment plants were replaced in the growth chamber and were cultivated for another 27 h in pollutant-free air. In a second set of experiments ozone was applied during the whole incubation period. After different time points, starting with the beginning of the ozone treatment, leaves 3 + 4 were harvested and the mid-veins were removed. The leaves were frozen with liquid nitrogen and stored at -75 °C until use for RNA extraction.

RNA extraction and blotting

Frozen leaves from one time point were ground to a fine powder using a mortar and pestle and 0.5 g of the powder was used for total RNA extraction. RNA was isolated using the guanidine hydrochloride method as described [14]. RNA was fractionated in 1.5% agarose-formaldehyde gels and then electroblotted onto a GeneScreen nylon membrane (NEN, Dreieich, FRG) as described [15]. RNA was fixed onto the membrane by UV light cross-linking (Stratagene, Heidelberg, FRG).

Hybridization

Pre-hybridization and hybridization was carried out in the presence of the corresponding labelled specific probe [14]. Blots were washed with 2 $\times$ SSC at room temperature for 5 min, then with 2 $\times$ SSC containing 1% SDS at 65 °C for 30 min and finally with 0.2 $\times$ SSC at 65 °C for 15 min. For rehybridization blots were washed with 5 mM Tris-HCl (pH 8.0), 2 mM Na₂EDTA, 0.05% sodium pyrophosphate and 0.1 $\times$ Denhardt's solution at 65 °C for 3 h. In order to check that no hybridization bands remained on the membranes, the blots were prolonged exposed to X-ray films before a rehybridization was carried out.

Slot blot hybridization

RNA dissolved in $1.5 \times \text{SSC}$ containing 50% formamide and 6.5% formaldehyde was heated for 10 min at 68 °C, immediately transferred to ice and then diluted with 1 volume of $20 \times \text{SSC}$. For each time point different amounts of RNA (0.5, 1.0, 2.0 and 5.0 μg) were blotted onto a nylon membrane using a slot blot apparatus and reduced air pressure. The total amount of RNA was adjusted to 5 μg with tRNA from yeast. Slots were washed twice with 0.5 ml $10 \times \text{SSC}$. RNA fixing and hybridization were as described above.

DNA probes for hybridization

The DNA inserts of the following clones were used for hybridization: pGL 43 (β -1,3-glucanase) [31]; pBSCHT15 (acidic chitinase PR-Q) [33]; pSAc 3 (actin) [40]; pCHN 50 (basic chitinase) [41]; pBS-PR1010 (PR-1b protein) (J. Ryals, unpublished). Probes were made by cutting plasmids with a suitable restriction enzyme. Vector and insert DNA were separated by electrophoresis through low-melting-point agarose gels. The DNA probes were labelled with ^{32}P -dCTP using a random-primed DNA labelling kit (Boehringer, Mannheim, FRG).

Autoradiography and quantification

Blots were autoradiographed at -75°C on pre-flashed Fuji X-ray films, using a Hyperscreen intensifier (Amersham, Braunschweig, FRG). Results were quantified by scanning the films with an ELSCRIPT 400 densitometer (Hirschmann, Unterhaching, FRG). The optical density was between 0.5 and 2.5. The linear range of the film was analysed by a comparison of dilution series of known amounts of radioactivity with the corresponding optical densities of the autoradiogram [43]. For each experimental series the highest mRNA level was set as 100% and other values are given relative to this value.

Results

Ozone-induced leaf injury

In accordance with earlier findings [21, 25], tobacco plants of cv. Bel W3 treated for 5 h with 0.15 $\mu\text{l/l}$ ozone showed foliar necrotic lesions, which developed between 15 and 72 h after exposure, whereas no visible injury occurred in the ozone-tolerant cv. Bel B. Continuous ozone treatment of cv. Bel W3 for 32 h resulted in an increased leaf damage (80%), compared to a single 5 h pulse (20%).

β -1,3 Glucanase mRNA accumulation

Total RNA isolated from leaf tissue was electrophoresed on 1.5% agarose-formaldehyde gels. Discrete ribosomal RNA bands, stained with ethidium bromide, indicated the intactness of the preparations (data not shown). To precisely correlate changes in mRNA levels to the ozone treatment, the data presented here are the results of tobacco from one planting. Two additional independent sets of experiments have been carried out with results corroborating those described.

Little or no β -1,3-glucanase mRNA was detectable in unfumigated tobacco plants. When cv. Bel W3 plants were exposed to ozone, a dramatic increase in the level of β -1,3-glucanase mRNA could be observed 1–2 h after the onset of fumigation, which was set off after 5 h (Figs. 1, 2). After 22 h (17 h after withdrawal of ozone), the β -1,3-glucanase mRNA level decreased. Since the increase in β -1,3-glucanase mRNA was measured in relation to total RNA, we considered the possibility that observed changes were the result of a non-specific shift in the relative proportions of ribosomal RNA and mRNA. To test this possibility, the β -1,3-glucanase probe was removed and the filter was reprobed with an actin clone from soybean. Actin is a 'housekeeping protein' of plants, and is often assumed to be constitutively expressed. The actin probe hybridized to two bands in tobacco and there was almost no difference during ozone treatment, although large

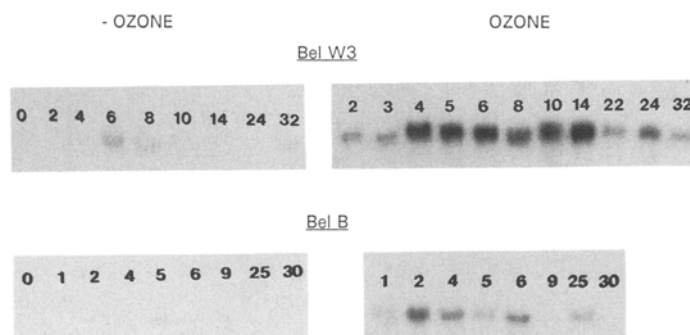


Fig. 1. Northern blotting of total RNA (5 µg) of ozone treated (+) and control (-) tobacco plants cv Bel W3 and Bel B. Plants were exposed to 0.15 µl/l ozone or pollutant-free air for 5 h and then further incubated in clean air. Hybridization was carried out with a labelled DNA probe for the β -1,3-glucanase gene (pGL 43). Numbers indicate the time (h) after the start of the experiment.

changes were visible for β -1,3-glucanase mRNA (Fig. 2). In results from control plants without ozone application, a slight increase of β -1,3-glucanase mRNA was noted 6–8 h after the onset of incubation. This appeared to result from transferring the plants from the growth chamber to the fumigation chambers and back to the growth chamber.

The transient induction of β -1,3-glucanase mRNA was analysed over a period of 32 h by the slot blot hybridization technique. Results are expressed as a percentage of maximum hybridization, setting the highest value as 100%. The

amount of β -1,3-glucanase mRNA increased with no obvious lag phase during the exposure period and remained high up to 15 h, then returned to lower levels (about 20–40%) during further cultivation in clean air (Fig. 3).

In contrast to this transient induction of β -1,3-glucanase mRNA after a single ozone pulse, a high mRNA level was evident when cv. Bel W3 was fumigated with ozone over the whole incubation period (Fig. 2). Large amounts of β -1,3-glucanase mRNA were detectable even after 49 h.

When the ozone-tolerant cv. Bel B was fumigated with 0.15 µl/l ozone for 5 h, β -1,3-glucanase mRNA was induced within 2 h. Its accumulation was maximal during the fumigation period and subsequently decreased to low basal levels and was hardly detectable during further incubation (Fig. 1). This was in contrast to transcript accumulation in cv. Bel W3, where high amounts of β -1,3-glucanase mRNA were still present up to 14 h (Fig. 1). The time-dependent accumulation and decrease is shown in Fig. 3. In comparison to cv. Bel W3 the maximum hybridization signal was reduced by a factor of 2.5. A β -1,3-glucanase mRNA amount of about 30–50% was reached during ozone treatment, but this level dropped to 0–10% after withdrawal of ozone.

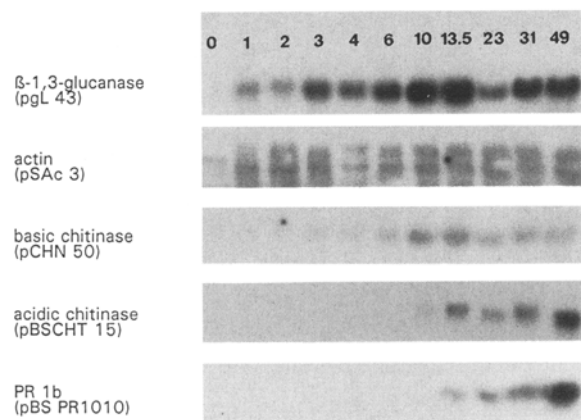


Fig. 2. Northern blotting of total RNA (5 µg) of ozone treated tobacco plants cv. Bel W3. Plants were exposed to continuous ozone (0.15 µl/l) for the time (in hours) indicated. Hybridization was carried out with the indicated labelled DNA probes.

Basic chitinase mRNA accumulation

The kinetics of accumulation of basic chitinase mRNA in cv. Bel W3 was different from that of

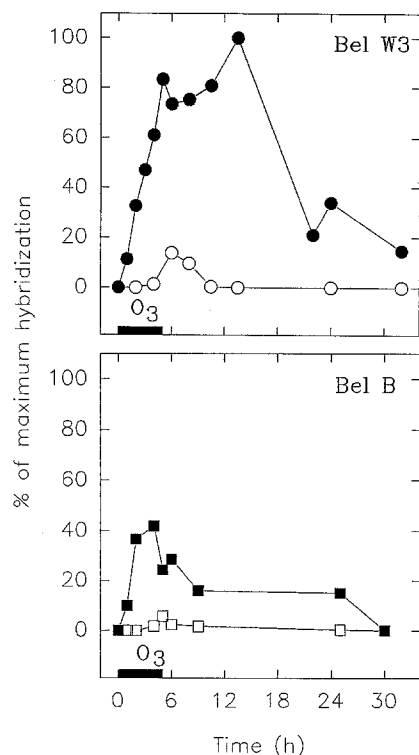


Fig. 3. Time course of changes in β -1,3-glucanase mRNA level in ozone treated and control tobacco plants. Plants cv Bel W3 and Bel B were treated with $0.15 \mu\text{l/l}$ ozone for 5 h (●, ■) or received ozone-free air (○, □). Blots of autoradiograms were scanned and the highest value set as 100%.

β -1,3-glucanase mRNA. A basal level of basic chitinase mRNA was visible in unfumigated tobacco plants (Fig. 4). After 4 h of ozone application an increase occurred and the mRNA amount

remained almost constant during fumigation and subsequent cultivation in pollutant-free air (Fig. 4). Even control treatment resulted in a considerable increase of basic chitinase mRNA, which appeared to result from transferring the plants to the fumigation chambers, similar as for the β -1,3-glucanase. Therefore the absolute value of induction of basic chitinase mRNA is much lower than for the β -1,3-glucanase mRNA (compare Figs. 1 and 4).

Continuous ozone treatment of cv. Bel W3 also resulted in a slight increase in basic chitinase mRNA levels during the first hours of treatment. Upon continuous fumigation a further increase was evident, reaching the highest amounts of basic chitinase mRNA after 13.5 h. After this time the level of mRNA was nearly constant (Fig. 2).

Tobacco cv. Bel B showed an increase of basic chitinase mRNA after 1 h of fumigation and still had high levels 30 h after the onset of fumigation (25 h after withdrawal of ozone) (Fig. 4). The time course of basic chitinase mRNA induction in control cv. Bel B was comparable to the control results obtained for β -1,3-glucanase mRNA in cvs. Bel B and Bel W3 and also with basic chitinase mRNA in cv. Bel W3 (compare Figs. 1 and 4).

Acidic chitinase mRNA accumulation

Basic β -1,3-glucanase and chitinase isoforms of tobacco are mainly located in the vacuole [3, 23].

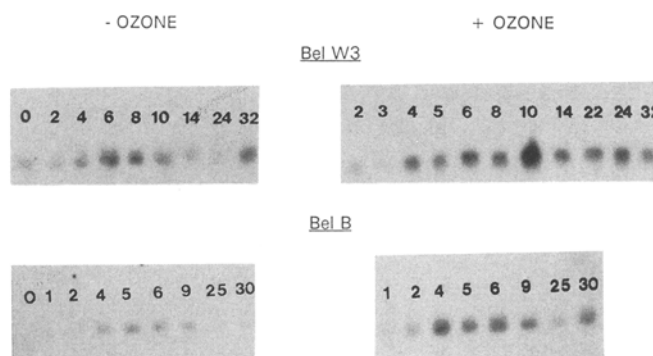


Fig. 4. Northern blot analysis of $5 \mu\text{g}$ RNA isolated from ozone-treated (5 h) (+) or non-treated (-) tobacco plants cv Bel W3 and Bel B. Hybridization was carried out with a labelled DNA, specific for basic chitinase (pCHN 50). Numbers indicate the time (h) after the start of the experiment.

The acidic chitinase is located intercellularly in the apoplast [3, 23], which is the primary target site for gaseous pollutants. We therefore analysed the level of this mRNA species. Both tobacco cultivars showed no detectable amounts of acidic chitinase mRNA after 5 h of ozone treatment. However, when cv. Bel W3 was exposed continuously to ozone, an increase of the mRNA level was seen 13.5 h after onset of fumigation (Fig. 2). After 2 days of ozone treatment a further increase was obvious (Fig. 2).

PR-1b mRNA accumulation

The accumulation of the PR-1b mRNA upon ozone treatment of Bel W3 and Bel B was similar to that found for the acidic chitinase (Fig. 2). A weak hybridization signal appeared only after prolonged ozone treatment (14 h) of cv. Bel W3. After 2 days of ozone fumigation the amount of PR-1b mRNA became more pronounced. These results indicate that ozone-induced gene expression of PR-1b as well as acidic chitinase is not a primary event caused by ozone fumigation compared to β -1,3-glucanase and basic chitinase.

Discussion

The expression of plant genes encoding hydrolases upon attack by pathogenic organisms has been under intensive study [10]. In many higher plants β -1,3-glucanase and chitinase mRNA are induced upon pathogen attack, after elicitor treatment and in response to ethylene [3, 4, 6, 11]. In this report we describe several new findings. First, ozone fumigation results in an induction of expression for several PR genes. Second, the rapidity of the increase in β -1,3-glucanase mRNA (Figs. 1 and 2) demonstrates a very early response. Third, the two tobacco cultivars studied show a different expression pattern of the hydrolases analysed.

We have previously shown an increase of enzyme activities of β -1,3-glucanase and chitinase

upon ozone treatment of tobacco cv. Bel W3 and Bel B, and gave preliminary evidence for an induction of β -1,3-glucanase mRNA [39]. In order to get a better insight into the complex mechanisms of interaction between air pollutants and gene expression, the ozone-induced transcript accumulation of different hydrolases was analysed. The data indicated that treatment of tobacco plants with ozone resulted in increased levels of β -1,3-glucanase and basic chitinase mRNA. One to two hours of ozone fumigation were sufficient to induce β -1,3-glucanase mRNA. This increase in mRNA level preceded an enhanced enzyme activity of β -1,3-glucanase, which reached near-maximum values after 10 h [39]. Thus β -1,3-glucanase mRNA accumulation took place long before the onset of lesion formation in cv. Bel W3 and without any visible injury in cv. Bel B. An increase of β -1,3-glucanase mRNA also occurred in the hypersensitive reaction of *Nicotiana tabacum* cv. Samsun NN to tobacco mosaic virus (TMV) two days after infection [47]. In tobacco tissue cultures a delayed increase of β -1,3-glucanase mRNA has been reported after depletion of auxins and cytokinins [31]. In contrast to these reports, ozone treatment resulted in a very rapid increase of β -1,3-glucanase mRNA (Fig. 2). This indicated that ozone affected gene expression just as quickly as fungal elicitors or UV treatment [7, 24], or perhaps even as rapidly as the phytochrome system [8, 37].

It was already known that ethylene can induce β -1,3-glucanase and basic chitinase in a variety of plants [4, 6, 16, 28, 30, 44] and that translatable mRNA increased within 2 h in response to ethylene treatment [46]. The ozone-induced increase of β -1,3-glucanase mRNA was similar to this ethylene-induced increase (Figs. 1, 3). Ozone is known to induce ethylene formation and a correlation between plant ozone tolerance and ozone-induced ethylene formation has been shown [25, 29]. In tobacco cv. Bel W3 a transient increase of ethylene formation is measured after one hour of ozone exposure [25]. This is consistent with the observed increase in β -1,3-glucanase mRNA in the same cultivar (Figs. 1, 2). In the ozone-tolerant cultivar Bel B we also observe an

increase in β -1,3-glucanase mRNA upon ozone fumigation, although this increase is not as drastic as that in cv. Bel W3. In contrast to cv. Bel W3, there was only a slight increase in ethylene production in cv. Bel B after ozone fumigation [25]. This behaviour suggests release of endogenous elicitors, to mediate the effect of ozone on gene expression. Similarly, in case of local induction of *win2* mRNA by wounding, transcription does not have an absolute requirement for ethylene, although ethylene is necessary for high levels of expression [50]. The low level of stress ethylene formed in cv. Bel B may have been sufficient for the observed induction of this gene. It has to be stressed, however, that the rapidity of induction of β -1,3-glucanase mRNA implies that this is not an indirect effect, but rather an early component of plant defence reactions.

In tobacco cv. Bel W3 an increased level of β -1,3-glucanase was still obvious 20 h after withdrawal of ozone (Fig. 1). As stress ethylene production decreased to low levels after 5 h of ozone fumigation [25], a continuous high ethylene level was obviously not necessary for β -1,3-glucanase gene expression. This is in contrast to results obtained with bean plants, where ethylene treatment strongly increased the level of β -1,3-glucanase mRNA after 2 h. However, withdrawal of ethylene resulted in a rapid disappearance of the mRNA within 4 h [46]. This difference in behaviour may be due to the development of necrosis in tobacco cv. Bel W3.

For the basic chitinase mRNA we observed a similar increase in cv. Bel W3, which showed a good correlation to the increased enzyme activity [39]. The induction was delayed by 4 h and was not as strong as for the β -1,3-glucanase mRNA (compare Figs. 1 and 4). This finding is in contrast to reports on simultaneous increase of β -1,3-glucanase and chitinase upon TMV infection or ethylene treatment [33, 46, 47], or in wounded tobacco explants, where an increase in chitinase mRNA and subsequently in β -1,3-glucanase mRNA was found [32]. As the genome of tobacco contains a small multigene family for basic glucanase and basic chitinase [3, 26, 49], these small differences in the timing of induction might

be due to the activation of different genes of the family by the different stimuli.

In further experiments the mRNA levels of several hydrolases were analysed during continuous ozone fumigation (Fig. 2). In contrast to a single ozone pulse of 5 h, a continuous high level of β -1,3-glucanase mRNA and also an increased level of basic chitinase mRNA were detected, even after 2 d of exposure (compare Figs. 1 and 2). This different effect may result from a complex interaction of multiple factors.

Furthermore, under continuous ozone application, an increase in acidic chitinase mRNA and PR-1b mRNA, which is assumed to code for an acidic protein [9] was seen only after 14 h (Fig. 2). These genes were not induced after a single ozone pulse of 5 h. This is consistent with ethylene induced gene expression in tobacco cv. Samsun NN and cv. Petit Havanna, and in *Arabidopsis thaliana*, where the induction of defence genes appeared to be confined to the members encoding basic isoforms of enzymes [5, 30, 35]. The ethylene-induced basic isoforms of glucanase and chitinase are predominantly located in the vacuole [23, 28], whereas the acidic isoforms are secreted into the extracellular space [23]. As the induction of both enzymes by a single ozone pulse occurred intracellularly [39] and mRNA levels of the basic isoforms were elevated (Figs. 1, 2), it is very likely, that ozone and ethylene treatment results in the expression of similar genes. However, for acidic PR proteins an ethylene-independent inductive pathway in tobacco cv. Samsun NN has been shown [27]. Therefore at least the late increase in acidic chitinase mRNA and PR-1b mRNA upon ozone treatment could result from an activation of such a pathway.

In the ozone-resistant cultivar Bel B we observed an increased expression of basic chitinase mRNA upon ozone treatment (Fig. 4) which is in accordance with increased chitinase activity [39]. In contrast to cv. Bel W3, the induction of basic chitinase mRNA seems to be more pronounced than that of the β -1,3-glucanase mRNA, although the time course is similar (compare Figs. 1 and 4). The results, summarized in Table 1, indicate that cv. Bel B and cv. Bel W3 show a different accu-

Table 1. Induction of PR gene expression in tobacco cvs. Bel W3 and Bel B. Tobacco plants were treated with an ozone pulse (0.15 $\mu\text{l/l}$, 5 h).

	β -1,3-glucanase		Basic chitinase		Acidic chitinase	PR-1b
	mRNA level	enzyme activity	mRNA level	enzyme activity	mRNA level	mRNA level
Bel W3	+	+	+	+	–	–
Bel B	+	+	+	+	–	–

++++, strong induction; +, weak induction; –, no induction. The induction of enzyme activities was taken from Schrundner *et al.* [39].

mulation of PR mRNAs upon a single ozone pulse.

Oxidative stress thus resulted in a gene expression similar to that described for pathogen attack, wounding, and ethylene or elicitor treatment. Recent evidence points to significant oxygen radical formation in some plant tissue in response to pathogenic challenge [42]. In *Nicotiana tabacum* leaf discs a TMV-stimulated and in cell cultures of *Glycine max* an elicitor-stimulated oxidative burst have been found [1, 2, 13]. Ozone and ozone-derived oxy radicals, which are not removed by scavengers in the apoplastic fluid, can react with the membrane [18]. As a result, a signal pathway may be induced, leading to defence reactions. The very rapid response of β -1,3-glucanase mRNA and the diverse responses of the PR mRNAs reported in this paper can be used as a basis for further studies in elucidating this signal transduction chain.

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