



Ozone-induced gene expression occurs via ethylene-dependent and -independent signalling

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Received 9 January 2002; accepted in revised form 31 July 2002

Key words: ethylene, gene regulation, ozone, pathogenesis-related, promoter, resveratrol, stilbene

Abstract

Recent studies suggest that ethylene is involved in signalling ozone-induced gene expression. We show here that application of ozone increased glucuronidase (GUS) expression of chimeric reporter genes regulated by the promoters of the tobacco class I β -1,3-glucanases (*GLB* and *Gln2*) and the grapevine resveratrol synthase (*Vst1*) genes in transgenic tobacco leaves. 5'-deletion analysis of the class I β -1,3-glucanase promoter revealed that ozone-induced gene regulation is mainly mediated by the distal enhancer region containing the positively acting ethylene-responsive element (ERE). In addition, application of 1-methylcyclopropene (1-MCP), an inhibitor of ethylene action, blocked ozone-induced class I β -1,3-glucanase promoter activity. Enhancer activity and ethylene-responsiveness depended on the integrity of the GCC boxes, *cis*-acting elements present in the ERE of the class I β -1,3-glucanase and the basic-type pathogenesis-related PR-1 protein (*PRB-1b*) gene promoters. The minimal *PRB-1b* promoter containing only the ERE with intact GCC boxes, was sufficient to confer 10-fold ozone inducibility to a GUS-reporter gene, while a substitution mutation in the GCC box abolished ozone responsiveness. The ERE region of the class I β -1,3-glucanase promoter containing two intact GCC boxes conferred strong ozone inducibility to a minimal cauliflower mosaic virus (CaMV) 35S RNA promoter, whereas two single-base substitution in the GCC boxes resulted in a complete loss of ozone inducibility. Taken together, these data strongly suggest that ethylene is signalling ozone-induced expression of class I β -1,3-glucanase and *PRB-1b* genes. Promoter analysis of the stilbene synthase *Vst1* gene unravelled different regions for ozone and ethylene-responsiveness. Application of 1-MCP blocked ethylene-induced *Vst1* induction, but ozone induction was not affected. This shows that ozone-induced gene expression occurs via at least two different signalling mechanisms and suggests an additional ethylene independent signalling pathway for ozone-induced expression of genes involved in phytoalexin biosynthesis.

Abbreviations: CaMV, cauliflower mosaic virus; ERE, ethylene-responsive element; EREBP, ERE binding protein; *GLB*, class I β -1,3-glucanase B gene; *Gln2*, tobacco β -1,3-glucanase gene; GUS, glucuronidase; HR, hypersensitive response; 1-MCP, 1-methylcyclopropene; *PRB-1b*, basic-type pathogenesis-related protein gene; ROI, reactive oxygen intermediate; SA, salicylic acid; STS, stilbene synthase; *Vst1*, resveratrol synthase gene

Introduction

Stratospheric ozone (O₃) protects life from detrimental ultraviolet-B radiation, but tropospheric O₃ is a serious world-wide pollutant (Krupa, 2000). The phytotoxic effects of O₃ were recognized as early as 1950,

and subsequent studies have shown the damaging effects of O₃ at the physiological, biochemical and molecular biological level (Runeckles and Chevone, 1992; Kangasjärvi *et al.*, 1994; Sandermann, 1996; Sandermann *et al.*, 1998; Rao *et al.*, 2000a). Studies with different plant species have shown an ozone-

induced transcript accumulation of several defence-related genes (Ernst *et al.*, 1992; Eckey-Kaltenbach *et al.*, 1994; Sharma and Davis, 1997).

The specific mechanisms by which O₃ causes changes in plant gene expression are not known, but it is generally accepted that O₃ initiates an oxidative burst and an active production of reactive oxygen intermediates (ROIs) (Schraudner *et al.*, 1998; Pellinen *et al.*, 1999; Rao and Davis, 1999). The localized lesions observed in O₃-treated leaves are similar to those of the hypersensitive response (HR) in incompatible plant-pathogen interactions (Lamb and Dixon, 1997; Schraudner *et al.*, 1998). Furthermore, the rapid generation of ROIs and changes in transcript accumulation of defence-related genes resemble the responses to pathogen attack (Sandermann, 1996; Sharma *et al.*, 1996; Schraudner *et al.*, 1998). Based on these similarities several signal molecules like ethylene (Mehlhorn and Wellburn, 1987; Pell *et al.*, 1997; Tuomainen *et al.*, 1997), jasmonic acid (Örvar *et al.*, 1997) and salicylic acid (SA) (Sharma *et al.*, 1996; Rao and Davis, 1999) have been hypothesized to act as a second or third messenger for O₃-induced gene expression. However, opposite roles for ethylene and jasmonic acid signalling in cell death production, as well as insensitivity to salicylic acid and jasmonic acid, have been shown for ozone-sensitive *Arabidopsis* and poplar, respectively (Koch *et al.*, 2000; Overmyer *et al.*, 2000; Rao *et al.* 2000b). These various signal molecules often appear to interact, resulting in a dynamic response to biotic, as well as abiotic stimuli (Yang *et al.*, 1997; Reymond and Farmer, 1998; Scheel, 1998; Maleck and Dietrich, 1999; Smallwood *et al.*, 1999).

If both O₃ and pathogen challenge induce the expression of identical genes, it could be predicted that similar regulatory components of the promoter regions are influenced by the two stimuli. The transcriptional control of pathogenesis-related (PR) proteins is well studied and several *cis*-acting elements have been described (Rushton and Somssich, 1998; Buchel and Linthorst, 1999; Leubner-Metzger and Meins, 1999). Deletion analysis of the promoters of class I β -1,3-glucanase (*GLB* and *Gln2*) and the *PRB-1b* gene of tobacco revealed ethylene-responsive elements (EREs) that are essential for ethylene enhanced gene expression (Hart *et al.*, 1993; Vögeli-Lange *et al.* 1994; Ohme-Takagi and Shinshi, 1995; Sessa *et al.*, 1995). Enhancer activity and ethylene responsiveness of these promoters depend on the integrity of a GCC box within the ERE, which is well conserved in several

ethylene-inducible genes. The GCC box is the binding site of ERE-binding proteins (EREBPs), which are transcription factors mediating these responses to ethylene and fungal elicitors (Leubner-Metzger *et al.*, 1998; Yamamoto *et al.* 1999). The rapid formation of stress-ethylene is a well-known response by O₃ (Mehlhorn and Wellburn, 1987; Langebartels *et al.*, 1991), and an induction of β -1,3-glucanase and PR1b mRNA was found in tobacco (Ernst *et al.*, 1992; Schraudner *et al.*, 1992). Similarly transcripts of other PR proteins were found to be induced by ozone in parsley, birch and *Arabidopsis* (Eckey-Kaltenbach *et al.*, 1997; Pääkkönen *et al.*, 1998; Heidenreich *et al.*, 1999). This suggests that these different stresses are linked to common *cis/trans* elements.

The defence-related proteins induced upon pathogen attack or O₃ treatment include a second set of enzymes involved in phytoalexin production (Hahlbrock and Scheel, 1989; Rosemann *et al.*, 1991; Fischer and Hain, 1994; Preisig-Müller *et al.*, 1999; Chiron *et al.*, 2000a). Stilbene phytoalexins have so far been detected in only few plant families and are synthesized by stilbene synthases (STS). The biosynthesis of stilbene phytoalexins is one of the most sensitive ozone responses in pine and grapevine, and initiated at the level of transcription (Schubert *et al.*, 1997; Zinser *et al.*, 1998; Chiron *et al.*, 2000a, b). Promoter deletion analysis of the grapevine resveratrol gene (*Vst1*) demonstrated that an O₃-responsive region differs from the basal pathogen-responsive sequence (Grimmig *et al.*, 1997; Schubert *et al.*, 1997). However, an ethylene-responsive like element and ethylene-responsive enhancer-like elements have been described in the *Vst1* promoter (Ernst *et al.*, 1999), indicating again similar *cis/trans* elements in O₃/ethylene-mediated gene regulation.

In the present study we have examined the role of O₃ and ethylene in the transcriptional regulation of several class I β -1,3-glucanase genes, as well as the *Vst1* gene. Using deletion constructs of the *GLB* promoter, point mutations of the GCC box in the *PRB-1b* promoter, as well as tandem copies of GCC boxes of *Gln2* promoter we demonstrate that ethylene is involved in signalling O₃-induced regulation of these genes. To further define O₃-induced gene regulation we examined the regulation of *Vst1* and *GLB* promoters in transgenic tobacco plants by the application of 1-methylcyclopropene (1-MCP), an ethylene-action inhibitor (Sisler and Serek, 1997). From these results obtained we propose that O₃-induced gene regulation occurs at least via two distinct transduction pathways.

Materials and methods

Stilbene synthase promoter construct

For stilbene synthase promoter analysis in transgenic tobacco (*Nicotiana tabacum* cv. Petit Havana SR1), upstream sequences from the grapevine *Vst1* gene (Wiese *et al.*, 1994) (−1500 to +87), comprising the 5′ non-coding region and the first coding nucleotides, was translationally fused to the GUS reporter gene as previously described (Fischer, 1994; Schubert *et al.*, 1997).

β-1,3-Glucanase promoter constructs

Promoter analysis of the tobacco class I β-1,3-glucanase B gene (*GLB*) used upstream sequences from −1630 to +6 transcriptionally fused to the GUS reporter gene and a series of 5′ unilateral deletions as described (Vögeli-Lange *et al.*, 1994; Leubner-Metzger *et al.*, 1998). Two high-, two medium- and one low-expressing transformants of *Nicotiana tabacum* cv. Havana 425 plants were used as described (Vögeli-Lange *et al.*, 1994).

PRB-1b promoter constructs

Promoter constructs of the *PRB-1b* gene encoding a basic type pathogenesis-related protein have been described (Meller *et al.*, 1993; Sessa *et al.*, 1995). The constructs used in our study include a 213 bp minimal ethylene inducible promoter and two mutant forms, Gm1 and Gm2, disrupting an G-box like motif and an ethylene responsive GCC box, respectively (Sessa *et al.*, 1995). Transformants of *N. tabacum* cv. Samsun NN were used (Sessa *et al.*, 1995).

GCC and mGCC constructs

A synthetic GCC fragment was used as described (Ohme-Takagi and Shinshi, 1995) using 5′-upstream sequences (−1164 to −1118) of the tobacco β-1,3-glucanase *Gln2* gene (Ohme-Takagi and Shinshi, 1990). In the mutated fragment mGCC two single-base substitutions of the GCC motifs are known to abolish EREBP binding and ethylene inducibility (Ohme-Takagi and Shinshi, 1995). Both fragments were dimerized in tandem and fused to minimal promoter sequences (−46 to +8) of the cauliflower mosaic virus (CaMV) 35S gene regulating a GUS reporter transgene in *N. tabacum* cv. BY4 (Ohme-Takagi and Shinshi, 1995).

Exposure to ozone

Tobacco plants were exposed to a constant stream of air with O₃ in Plexiglas boxes (730 l) placed in an environmental growth chamber at 25/20 day/night cycle with a 12 h photoperiod (0.15 mE m^{−2} s^{−1}) and 65–70% relative humidity. O₃ was generated by electrical discharge in dry oxygen, and the desired O₃ concentration was computer-controlled together with the Photomet ozone analyzer CSI 3100 (Columbia Scientific Industries, Austin, TX; Langebartels *et al.*, 1991). After O₃ treatment, the plants were incubated in pollutant-free air.

Exposure to ethylene

For ethylene treatment, plants were placed in sealed Plexiglas boxes and ethylene was supplied with a syringe. Ethylene concentrations in the exposure cuvet were determined with a gas chromatograph equipped with a Poropak Q column and a flame ionization detector (Langebartels *et al.*, 1991).

Ethylene response inhibitor treatment

The ethylene response inhibitor 1-methylcyclopropene (1-MCP) was applied 12–14 h before the onset of O₃ or ethylene treatment. The plants were placed in sealed cuvettes and an appropriate amount of EthylBloc powder (Biotechnologies for Horticulture, USA; obtained from Laboratorium van der Sprong, Netherlands) containing 1-MCP was stirred in 0.75% KOH, 0.75% NaOH (20 ml/g EthylBloc) in a glass beaker placed inside the closed exposure cuvette.

GUS assay

Frozen leaf material was homogenized in GUS lysis buffer (3× v/w) containing 50 mM sodium phosphate buffer pH 7.0, 5 mM EDTA, 0.1% Triton X-100, 0.1% Sarcosyl and 20 mM 2-mercaptoethanol for 2 min by vigorous shaking (UniPrep from UniEquip, Martinsried, Germany). The extracts were tested fluorometrically for GUS activity as described (Jefferson, 1987) with 4 methylumbelliferyl-β-D-glucuronide (Biomol).

Results

Identification of ozone-responsive regions of the *GLB* and *PRB-1b* promoters

Pathogenesis-related proteins, such as class I β -1,3-glucanases and *PRB-1b*, as well as stilbene synthase belonging to the phenylpropanoid pathway have been shown to be induced by O_3 at the transcriptional level (Ernst *et al.*, 1992; Schubert *et al.*, 1997). Class I β -1,3-glucanase and *PRB-1b* are known to respond to ethylene as shown by GUS reporter expression experiments with regulatory promoter sequences in transgenic tobacco plants (Vögeli-Lange *et al.*, 1994; Sessa *et al.*, 1995). We compared the induction by ethylene (10 μ l/l) and O_3 (300 nl/l) and showed that the *GLB* and *PRB-1b* promoter responded, albeit to a lesser extent, to O_3 treatment (data not shown) confirming previous RNA blotting experiments (Ernst *et al.*, 1992). The ethylene-responsive regions of the *GLB* promoter have been identified earlier and showed that the distal sequences confer ethylene responsiveness (Vögeli-Lange *et al.*, 1994; Leubner-Metzger *et al.*, 1998). In contrast, the O_3 -responsive promoter regions have not been analysed.

In order to map O_3 -responsive sequences we tested the O_3 inducibility of the various 5'-deleted *GLB* promoter::GUS constructs used in the previous study for ethylene induction (for the structure of the constructs see Vögeli-Lange *et al.*, 1994). For each construct progeny from usually five individual transformant lines showing high, medium and low expression, and up to 4 individual plants for each line were used for testing. Data were expressed for each individual plant as fold induction of GUS activity measured in samples taken from opposite halves of leaf 3 (counting from the top of the plant with leaf no. 1 >8 cm) before and after fumigation with O_3 (Figure 1A). A second combined sample was taken from each plant from leaves 2 and 4 and fold induction of GUS activity was expressed compared to control sample taken from leaf 3 (Figure 1B). Wounding by the first sample withdrawal did not cause activation of the *GLB* promoter constructs in samples taken from the opposite leaf halves (control, data not shown). High O_3 -responsiveness was observed in transformants containing the -1630 and -1452 construct and dropped markedly upon further deletion (Figure 1) indicating the relevance of sequences between -1492 and -1193 for strong regulated expression, as has been shown for ethylene (Vögeli-Lange *et al.*, 1994; Leubner-Metzger

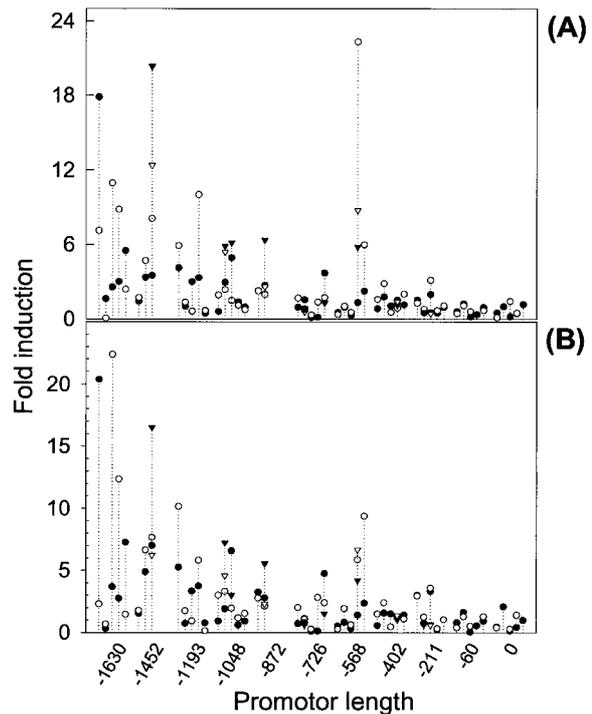


Figure 1. Determination of the O_3 -responsive region of the *GLB* promoter in transgenic tobacco plants. The length of the 5' unilaterally deleted promoter fragments derived from a -1630 to +6 promoter fragment of the tobacco class I β -1,3-glucanase B gene (*GLB*) is shown on the bottom. For each construct, several independent transformed lines were tested for ozone responsiveness. Usually 4 individual plants (\bullet , \circ , \blacktriangledown , \triangledown) of each transformant line and up to 5 lines for each construct were exposed for 24 h with 200 $nl\ l^{-1}$ O_3 . Control samples were taken from leaf 3 (counted from the top, with leaf no. 1 >8 cm) of individual plants immediately before exposure. After exposure samples were taken from opposite halves of leaf no. 3 (A), or combined samples from leaves no. 2 and 4 (B). In all cases an equal number of leaf discs were punched out of the leaves with a cork borer (2.5 cm \varnothing). Data are expressed as fold induction of GUS activity in corresponding opposite halves of leaf 3 from individual plants (\bullet , \circ , \blacktriangledown , \triangledown) (A), or as fold induction of leaf samples 2/4 to control sample leaf 3 (\bullet , \circ , \blacktriangledown , \triangledown) (B).

et al., 1998). Promoter -568 gave higher level of expression compared to promoter -726, suggesting that the deleted region contains negative elements. O_3 responsiveness was maintained down to -568 and was lost upon further deletion (Figure 1). Therefore, responsiveness of the *GLB* promoter towards ozone mirrors the pattern found previously for ethylene suggesting an intimate connection of ozone and ethylene in *GLB* regulation. Ethylene formation is an early response to O_3 (Sandermann, 1996) and may therefore contribute or be responsible for O_3 -induced gene expression. In order to ensure the role of stress ethylene

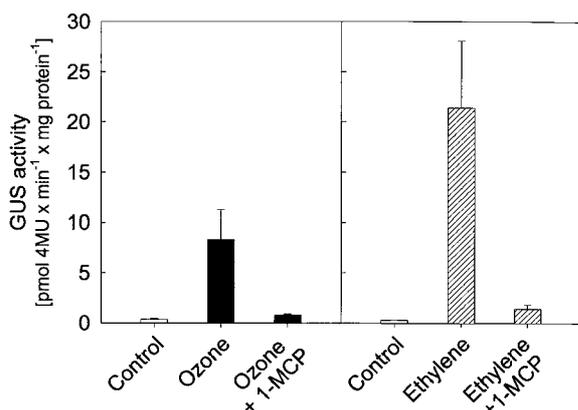


Figure 2. Effect of the ethylene response inhibitor 1-MCP on *GLB* promoter responsiveness towards O_3 and ethylene. Tobacco plants transformed with the 1.63 kb *GLB::GUS* construct were exposed overnight to 1-MCP (1 μ l/l) and then treated with O_3 (300 nl/l) or ethylene (10 μ l/l) for 8 h, while control plants were held in pollutant-free air after 1-MCP pretreatment. Samples for GUS activity measurement were taken immediately before and 24 h after onset of fumigation from opposite halves of a middle-aged leaf. Means \pm SE ($n = 3$).

in O_3 -induced gene expression we demonstrated that tobacco plants responded with a fast transient release of ethylene after ozone treatment (data not shown) corroborating similar results obtained with tomato plants (Tuomainen *et al.*, 1997).

The role of ethylene as downstream messenger of O_3 -induced *GLB* expression was tested with 1-MCP, a competitive inhibitor of ethylene receptors. As shown in Figure 2, the *GLB* promoter was induced by ethylene and, to a somewhat lesser extent, by O_3 . Application of 1-MCP completely blocked ethylene-, as well as O_3 -induced *GLB* promoter activity, indicating that stress ethylene perception is responsible for *GLB* induction by O_3 .

Ozone induction of the minimal ethylene-responsive PRB-1b promoter

The basic-type pathogenesis-related protein PRB-1b is activated at the transcriptional level by ethylene (Meller *et al.*, 1993). Promoter deletion and mutation experiments identified a GCC box and a G-box like motif within a -213 to -141 minimal ethylene-responsive promoter region (Sessa *et al.*, 1995). We used constructs of the minimal ethylene-responsive -213 *PRB-1b* promoter and compared its induction by O_3 to that of the -67 bp inactive promoter (TATA construct) in transgenic tobacco plants. As shown in Table 1, the minimal ethylene-responsive *PRB-1b* promoter was able to confer responsiveness towards

O_3 . Mutations in the G-box-like element decreased GUS activity, whereas responsiveness towards O_3 remained unaffected (Table 1). Disruption of the GCC box, however, severely reduced responsiveness towards O_3 , albeit not completely (Table 1). Again, these results demonstrate that ethylene can function as a downstream messenger of O_3 .

GCC-boxes alone are sufficient for ozone-responsive gene expression

The functional relevance of the GCC box for ethylene signalling had been demonstrated in experiments with a 47 bp fragment derived from the tobacco β -1,3-glucanase *Gln2* gene (Ohme-Takagi and Shinshi, 1990) containing a GCC box in duplicate (Ohme-Takagi and Shinshi, 1995). Independent transformant lines containing the $(GCC)_2::GUS$ -construct or the mutated GCC box (mGCC) were tested for their response towards O_3 (Table 1). The plants containing constructs with tandem copies of functional GCC boxes showed O_3 -induced GUS expression, whereas plants containing the mutated GCC-box construct showed no O_3 -inducible GUS activity (Table 1).

Role of ethylene in ozone-induced *Vst1* gene regulation

Promoter deletion experiments had identified an O_3 -responsive and pathogen-responsive region in the *Vst1* promoter between -430 and -280 and between -280 and -140, respectively (Grimmig *et al.*, 1997; Schubert *et al.*, 1997). In addition, further 5'-deletion analysis of the *Vst1* promoter revealed an ethylene-responsive region between -240 and -40 (Grimmig *et al.*, 2002). To further analyze the role of ethylene in O_3 -induced *Vst1* gene regulation, 1-MCP was tested as described for the *GLB* promoter. The *Vst1* promoter was induced by ethylene and to the same extent by O_3 (Figure 3). Application of 1-MCP just before ethylene treatment completely blocked the ethylene-induced *Vst1* induction. However, application of 1-MCP before O_3 fumigation did not influence O_3 -induced *Vst1* gene regulation (Figure 3).

Discussion

In recent years, O_3 has been recognized as an abiotic elicitor of many plant defence reactions (reviewed by Sandermann, 1996; Sharma and Davis, 1997; Sandermann *et al.*, 1998; Rao *et al.*, 2000a). Ethylene,

Table 1. Characterization of regulatory elements in the basic-type pathogenesis-related protein gene (*PRB-1b*) (Sessa *et al.*, 1995) and class I β -1,3-glucanase gene (*Gln2*) (Ohme-Tagaki and Shinshi, 1995) promoter involved in O₃ regulation. GCC (-213): minimal ethylene-inducible *PRB-1b* promoter; GCC (Gm1): mutation in the G box; GCC (Gm2): mutation in the GCC box; GCC (-67): ethylene-inactive deletion construct, containing the TATA box. (GCC)₂: ethylene-inducible tandem orientated GCC fragments of the *Gln2* gene; (mGCC)₂: a single 2 bp substitution in the tandem GCC fragments. The GCC boxes and G box are underlined. Base pair substitutions are indicated in bold. O₃ exposure was for 8 h with 300 nM. Samples for GUS activity measurements were removed 24 h after onset of fumigation. Several independent transformed lines were tested.

Promoter constructs	Sequence	Fold induction
<i>PRB-1b</i>		
GCC (-213)	ATGGCGGCTCTTATCTCACGTGATG	8.7
GCC (Gm1)	ATGGCGGCTCTTATCTCAATTGATG	10.8
GCC (Gm2)	ATGGCG T TAAATTATCTCACGTGATG	4.8
GCC (-67)	TATA box	1.7
<i>Gln2</i>		
(GCC) ₂	CATAAGAGCGCCACTN ₁₅ AAATAAGAGCGCCAT	6.8
(mGCC) ₂	CATAAGATCCTCCACTN ₁₅ AAATAAGATCCTCCAT	0.7

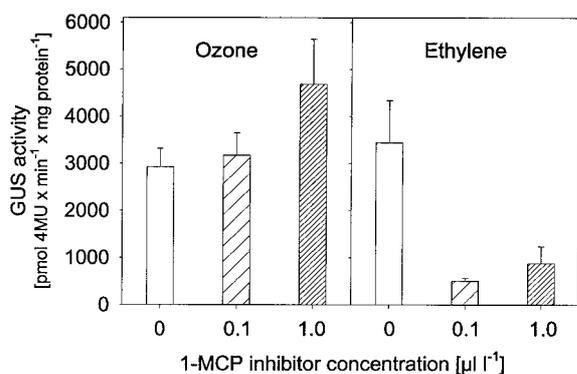


Figure 3. Effect of the ethylene response inhibitor (1-MCP) on *Vst1* promoter expression. Tobacco plants harbouring the 1.5 kb *Vst1::GUS* construct were exposed overnight to 1-MCP in an air-tight container. The plants were then transferred to clean air 1 h before onset of treatment with O₃ (300 nM) or ethylene (10 μM) for 8 h. Samples for GUS activity measurement were taken 12 h after onset of fumigation from middle-aged leaves. Means ± SE (n = 3).

SA and jasmonic acid, have been implicated as second or third messengers in signalling O₃ effects in plants (Sharma *et al.*, 1996; Örvar *et al.*, 1997; Tuomainen *et al.*, 1997; Overmyer *et al.*, 2000; Rao *et al.*, 2000b). The induction of *PR* genes, as well as of the *Vst1* gene by O₃ or ethylene is well documented (Ernst *et al.*, 1992; Meller *et al.*, 1993; Vögeli-Lange *et al.*, 1994; Eckey-Kaltenbach *et al.*, 1997; Grimmig *et al.*, 1997), and an ethylene-responsive GCC box has been characterized (Sessa *et al.*, 1995; Ohme-

Tagaki and Shinshi, 1995). Our present experiments, with transgenic plants harbouring the *GLB*, *PRB-1b* or *Vst1* promoter fused to the GUS reporter gene, confirm O₃-responsive gene expression. This indicates that the basic results obtained do not depend on the different genetic backgrounds of tobacco used, as has been similarly described for O₃ effects on *PR-1b* transcripts of *N. tabacum* Bel W3 (Ernst *et al.*, 1992) towards *PR* proteins in *N. tabacum* L. cv. Xanthi nc (Yalpani *et al.*, 1994) and on different transcripts of *N. plumbaginifolia* towards *N. tabacum* L. cv. PBD6 (Willekens *et al.*, 1994). However, differences between O₃-tolerant and O₃-sensitive species are known (Schraudner *et al.*, 1997).

Deletion analysis of the *GLB* promoter indicated that the distal -1452 to -1193 region is important for high-level GUS expression in response to ozone (Figure 1). This is in accordance with earlier reports on ethylene-induced *GLB* promoter constructs (Vögeli-Lange *et al.*, 1994; Leubner-Metzger *et al.*, 1998). This distal region contains the two copies of the GCC-box (Vögeli-Lange *et al.*, 1994; Leubner-Metzger *et al.*, 1998), known to be responsible for ethylene signalling. Further deletions resulted in reduced GUS activity. Interpretations of deletions shorter than -402 are difficult in view of the low expression level. In addition to the distal ERE region, the region from -568 to -402 also showed an increased level of expression upon O₃ treatment, indicating that negative elements

have been removed, as already shown for ethylene treatment (Vögeli-Lange *et al.*, 1994).

The GCC box is known to be sufficient for ethylene inducibility (Sessa *et al.*, 1995). Mutations affecting the G-box like element or the GCC box resulted in a strong decrease in *PRB-1b* promoter activity or completely abolished ethylene-responsiveness. This defines the conserved GCC-box motif as ethylene-specific *cis*-regulatory element (Sessa *et al.*, 1995). Our results concerning O₃ effects involving this GCC box provide the first evidence that this sequence participates in O₃-induced *PRB-1b* gene expression. Substitution of four bases resulted in loss of inducibility (Table 1). Alteration of the G-box motif located upstream of the GCC-box motif decreased *PRB-1b* expression in transgenic plants but not its overall inducibility (Table 1). This is, again, similar to gene expression induced by ethylene (Sessa *et al.*, 1995). Formation of stress ethylene upon O₃ treatment of tobacco is well known (Langebartels *et al.*, 1991) and has also been shown in this study (data not shown). This emphasizes our concept that the formation of ethylene signals O₃-induced basic *PR* gene regulation.

An additional argument that the GCC box is important in the regulation of ethylene-induced class I β -1,3-glucanase gene expression is given by earlier studies with a tandem GCC-box construct of the *Gln2* promoter (Ohme-Takagi and Shinshi, 1995). Our results showed that the 47 bp fragment containing two copies of the GCC box acts as a regulatory element that enhances O₃-induced gene expression in transgenic tobacco (Table 1). This provides further evidence for the biological function of ethylene in signalling O₃-induced gene regulation. Nuclear events in ethylene signalling pathways provide evidence that EREBP act as transcriptional activators of GCC-box-mediated gene expression (Leubner-Metzger *et al.*, 1998; Solano *et al.*, 1998) and that the corresponding genes are differentially regulated by ethylene and by abiotic stress conditions (Fujimoto *et al.*, 2000). An elicitor-responsive and ethylene-independent activation of GCC-box-mediated gene expression by phosphorylation/ dephosphorylation has been shown for the tobacco class I basic chitinase (Yamamoto *et al.*, 1999). To demonstrate the biological function of ethylene in signalling O₃-induced gene expression we used the ethylene inhibitor 1-MCP. Both ethylene and O₃ induction of the *GLB::GUS* promoter construct could be blocked completely by 1-MCP (Figure 2). This further demonstrates that ethylene is involved

and necessary in the O₃-induced pathway for class I β -1,3-glucanase genes.

In addition to O₃-induced basic *PR* gene expression, O₃-induced stilbene biosynthesis has been studied in detail (Zinser *et al.*, 1998; Chiron *et al.* 2000a, b). An O₃-responsive region of the *Vst1* promoter had been identified (Schubert *et al.*, 1997) and recently it could be demonstrated that this region differs from minimal sequences necessary for ethylene inducibility (Grimmig *et al.*, 2002). Surprisingly, application of 1-MCP did not block O₃-induced *Vst1* promoter-driven GUS activity, whereas ethylene-induced gene expression was blocked (Figure 3). This demonstrates an ethylene-independent O₃-signalling pathway of *Vst1* gene expression. It has been speculated that an incomplete inverse GCC-box present in the *Vst1* promoter might contribute to ethylene signalling (Schubert *et al.*, 1997). However, the data obtained for the mutated GCC-boxes and the importance of the GCC-box sequence (AGCCGCC) for EREBP binding (Fujimoto *et al.*, 2000) argue against this possibility. Ethylene-responsive enhancer elements have been identified in the *Vst1* promoter (Grimmig *et al.*, 1997; Ernst *et al.*, 1999), which could be confirmed by database searches (<http://sphinx.rug.ac.be:8080/PlantCARE/index.htm>). Therefore these elements could be sufficient for increased GUS activity upon ethylene treatment of transgenic tobacco plants.

Our studies have shown that an ethylene signalling pathway is involved in an O₃-induced regulation of class I β -1,3-glucanase genes. Stilbene synthase genes, involved in phytoalexin production, can also be induced by ethylene. However, for an O₃ induction of the *Vst1* gene an additional ethylene-independent pathway exists. Thus a tuneable regulation of gene expression upon O₃ exists, as has been described for defence gene expression (Dong, 1998; Reymond and Farmer, 1998; Maleck and Dietrich, 1999). Because O₃ activates different signalling pathways, a network of DNA-binding proteins selectively binding to *cis* elements will finally determine specific gene regulation. Thus no specific ozone-responsive *cis* element seems to be necessary. *Cis*-acting elements functioning as hormone-, elicitor- or antioxidative-response element are sufficient to trigger, in a modulated form, ozone-induced gene expression.

Acknowledgements

We thank Evi Kiefer for expert technical assistance. We thank G. Sessa and R. Fluhr (Weizmann Institute of Science, Rehovot, Israel) for providing the PRB-1b tobacco seeds and H. Shinshi (National Institute of Bioscience and Human-Technology, Tsukuba, Japan) for providing the GCC and mGCC tobacco seeds. This work was supported by a DAAD grant (A/97/07931) attributed to M.N.G.-P., EUROSILVA and the Deutsche Forschungsgemeinschaft (SFB 607).

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