Gene Induction of Stilbene Biosynthesis in Scots Pine in Response to Ozone Treatment, Wounding, and Fungal Infection[1]

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The *S*-adenosyl-L-methionine:pinosylvin-*O*-methyltransferase (*PMT*)[2] gene was sequenced from Scots pine (*Pinus sylvestris*). The open reading frame is arranged in two exons spaced by one 102-bp intron. Promoter regulatory elements such as two "CAAT" boxes and one "TATA" box were identified. Several cis-regulatory elements were recognized: stress-responsive elements (*Myb*-responsive elements) as well as G, H, and GC boxes. Moreover, elicitor-responsive elements (W boxes) and a sequence resembling the simian virus 40 enhancer core were found. In phloem and needles of control trees, the transcripts of stilbene synthase (*STS*) and *PMT* were hardly detectable. Increased ozone fumigation up to 0.3 μL L^{-1} enhanced the transcript level of *STS* and *PMT* in needles but not in healthy phloem. Wounding, e.g. mock inoculation, of stem-phloem was characterized by a transient increase in *STS* and *PMT* transcripts, which was more pronounced in the case of fungal inoculation. Combination of fungal-challenge or mock treatment with ozone resulted in a positive interaction at 0.3 μL L^{-1}. Scots pine stilbene formation appeared to be induced via *STS* and *PMT* gene expression upon ozone and fungal stress as well as wounding. The broad stress-responsiveness is in agreement with the range of various cis-acting elements detected in the *STS* and *PMT* promoters.

In pine species, the constitutive stilbenes pinosylvin (PS), and pinosylvin monomethylether (PSM) occur exclusively in the heartwood (Kindl, 1985). However, both compounds are induced in the sapwood, phloem, and needles as a response to wounding or fungal attack (Hart, 1981; Kindl, 1985; Richter and Wild, 1992; Lieutier et al., 1996).

An increasing amount of data shows that some of the stilbenoid constituents may function as phytoalexins in seedlings (Schöppner and Kindl, 1979; Derks and Creasy, 1989). The formation of stilbenes can be induced in young plants of Scots pine (*Pinus sylvestris*) by UV light and stress (Schöppner and Kindl, 1979) by a minimum of 4 h of ozone exposure at 0.2 μL L^{-1} in primary needles (Rosemann et al., 1991) and by fungal attack in the phloem of adult pine trees (Lieutier et al., 1996). Stilbenes have been classified either as constitutive protectants preventing the decay of wood by microorganisms or as induced phytoalexins that protect phloem against bark beetles and other insects and against fungi symbiotically associated with pine beetles (Hart, 1981; Lieutier et al., 1996). The pathway to methoxystilbenes originates from L-Phe and includes the activities of Phe ammonialyase (PAL), stilbene synthase (STS), and pinosylvin methyltransferase (PMT). It has been shown that STS activity is the limiting factor in the pathway leading to PS and PSM (Schanz et al., 1992). Changes in gene expression of *STS* and/or *PMT* conceivably are a critical point in the induced resistance by stilbenoids.

There is little information on the transcriptional effects of simultaneous application of different stressors in plants, but exposure of plants to UV-B radiation or O_3 may result in the induction of similar genes. In Scots pine seedlings, *STS* (and cinnamyl alcohol dehydrogenase) mRNA levels have been reported to increase upon O_3 fumigation (Zinser et al., 1998), and a pathogen-induced accumulation of *STS* mRNA has also been proven (Schwekendiek et al., 1992). The regulatory patterns are complex and may involve the differential induction of isoenzymes as described for PAL (Cramer et al., 1989) and the combinatorial interaction of several spatially separated promoter elements including exonic sequences (Hauffe et al., 1993).

After our previous report on the *PMT* cDNA sequence (Chiron et al., 1998) we now describe the pine *PMT* genomic sequence, including a 5′-flanking region and putative cis-regulatory elements that classify the enzyme as a typical member of the inducible phenylpropanoid pathway. Moreover, we report on

[1] This work was supported by the European Community (grant no. FAIR–BM–955721 to H.C.) and in part by EUROSILVA and the Deutsche Forschungsgemeinschaft (grant no. SFB 607).

[2] A patent application has been filed for the *PMT* gene (Deutsche Patentanmeldung no. 10006204.0).

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changed *STS* and *PMT* mRNA levels upon O_3 fumigation in needles as well as upon wounding and fungal inoculation in phloem. The latter treatments were also combined with O_3 fumigation to highlight the sequential action of transactive factors in the regulation of stilbene biosynthetic genes.

RESULTS

PMT Gene Organization

A total of 1,908-bp DNA sequence covering exonic, intronic, and flanking sequences of the *P. sylvestris PMT* gene has been determined. The full nucleotide sequence is shown in Figure 1. To facilitate sequence numbering the first nucleotide of the 5′-non-coding region of the cDNA has been set as +1. Comparison of the genomic sequence with the *PMT*-cDNA sequence (Chiron et al., 1998) resulted in 100% identity

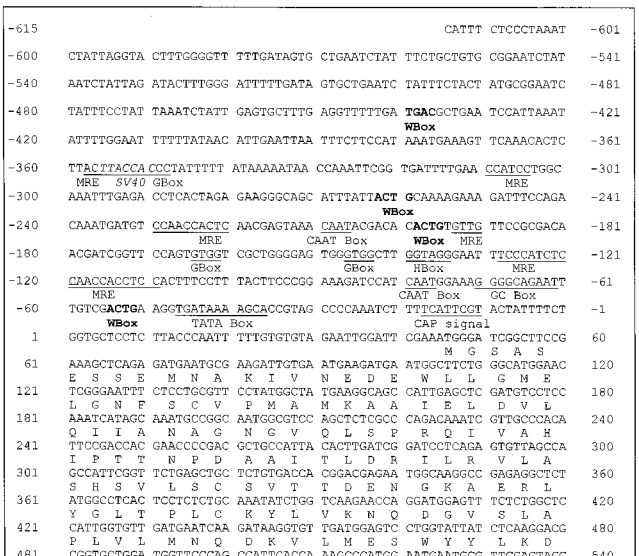

Figure 1. Nucleotide sequence of the *P. sylvestris PMT* gene and deduced protein sequence. The predicted transcription start site is noted as +1. Conserved eukaryotic promoter elements (CAP signal, TATA, CAAT, and GC boxes) and putative plant regulatory elements (G and H boxes), as well as MREs are underlined. Elicitor regulatory elements (W boxes) are bold and simian virus 40 (SV40) enhancer is italic.

in the 5′-non-coding region, whereas in the coding region two nucleotide exchanges were identified. They were located at positions 686 (T instead of C) and 953 (C instead of T) (numberings refer to Fig. 1). However, this resulted in no change of the deduced amino acid sequence. The *PMT* gene consisted of two coding exons and one intron region. The intron (102 bp) was in the range of the intron sizes commonly given (between 70 and thousands of nucleotides; Goodall and Filipowicz, 1991). The 5′-exon/intron and 3′-intron/exon boundaries conformed with the known GT/AG donor/acceptor site rule valid in both plants and animals (Brown, 1986). Upon closer inspection, the AG/GTA motif at the 5′-exon/intron splice junction was in accordance with the AG/GTAAG consensus. In addition, the intron contained the CAG/motif at the 3′-intron/exon splice junction in analogy to the TGCAG/G consensus for plant genes (Goodall and Filipowicz, 1991). The intron is AT rich (64%), which is essential for splicing (Goodall and Filipowicz, 1991). Inspection of upstream sequences showed conserved prokaryotic elements such as a catabolite activator protein (CAP) signal TCATTCGT at position −18, a TGATAAAAGCA motif at position −46 identified as a TATA box, and presumptive CAAT boxes located at positions −80 and −210.

The Scots pine *PMT* promoter contained potential cis-elements. *Myb*-responsive elements (MRE) are located at position −121, −129, −194, −230, −310, and −358. The element ACTTACCACCCT at position −358 matched in 8 of 12 positions the consensus sequence $^{T}/_{A}CT^{C}/_{A}ACCTA^{C}/_{A}C^{C}/_{A}$ in UV-light and fungal-elicitor-induced plant gene promoters of the phenylpropanoid pathway (Lois et al., 1989). At positions −121 and −230, the (A+C)-rich motifs CCAACCACCTCC and CCAACCACTC matched a second consensus motif ($CCA^{A}/_{C}C^{A}/_{T}AAC^{C}/_{T}CC$) in 10 and 9 of 12 positions, respectively (Lois et al., 1989). The element GTTG at position −194 was the inverse position of the core motif CAAC. Another motif, at position −129, TCCCATCTCC, matched in 9 of 10 positions the consensus of the box E ($^{A}/_{T}C$-$CC^{A}/_{T}{}^{T}/_{A}C^{A}/_{T}{}^{A}/_{T}{}^{G}/_{C}$), which appears to be conserved in stress-inducible phenylpropanoid gene promoters (Grimmig and Matern, 1997).

Other regulatory cis-acting elements known to flavonoid and stilbene biosynthetic genes were also detected: G-box-like motifs (GTGG at positions −147 and −165, CACC at position −353) (Faktor et al., 1996); H-box-like motif CCATCC in inverse orientation (GGTAGG at position −140); GC box, GGGGCA-GAAT (consensus $^{G}/_{T}GGGCGG^{G}/_{A}{}^{G}/_{A}{}^{C}/_{T}$), at position −72; elicitor-responsive elements, i.e. W boxes (ACTG at positions −55, −199, and −263, and TGAC at position −440) (Rushton and Somssich, 1998), and a SV40 enhancer core in inverse position (TTACCAC at position −356).

Stress Effects on Scots Pine

Single Ozone Treatment

In all reverse transcriptase (RT)-PCR experiments only single bands were detected at 1.15 kb for *PMT*, 1.17 kb for *STS*, and 0.8 kb for chlorophyll a/b-binding protein (*cab*). RT-PCR controls performed with cab primers showed a steady-state level of *cab* transcripts in non-ozone fumigated tissues, and a decrease of these transcripts in tissues exposed to 0.15 and 0.3 μL L^{-1} ozone (data not shown). In control trees exposed to ozone-free air, *STS* and *PMT* transcripts were hardly detectable in needles (Fig. 2) and phloem (Figs. 3 and 4A). Two 10-h periods of ozone fumigation were sufficient to dramatically increase *STS* and *PMT* transcript levels in needles of Scots pine trees (Fig. 2). Exposure to 0.15 μL L^{-1} ozone resulted in a first peak of *STS* transcripts after 6 h of exposure followed by another increase 48 h after the onset of fumigation, whereas *PMT* transcripts remained at the control level until 24 h after the beginning of the treatment and only then began to accumulate. Exposure to 0.3 μL L^{-1} ozone led to a further increase of both transcript levels and remained at a high level in the needles (Fig. 2). In contrast *STS* and *PMT* mRNA levels in phloem were not significantly affected by ozone (Figs. 3 and 4A).

Effects of Ozone Treatment on Wounding and Fungus Inoculation into Pine Phloem

A mock inoculation (Figs. 3 and 4B) led to the accumulation of both transcripts on d 5 (3 d after inoculation). *STS* mRNA displayed a progressive accumulation up to d 9 (120 ng ng^{-1} cDNA), then declined slowly, and was still at a high level after 16 d. *PMT* mRNA decreased progressively after d 5 (50 ng ng^{-1} cDNA) to reach the control level at the end of the experiment. The two kinetics in response to mock inoculation were strongly affected by a previous ozone treatment, and 0.15 μL L^{-1} ozone led to a dramatic decrease of *STS* mRNA response pattern. The *PMT* mRNA response curve was also lowered. A 0.3-μL L^{-1} ozone pretreatment resulted in the same 9-d peak of *STS* transcripts as mock inoculation alone but prolonged the accumulation until d 16. *PMT* transcripts showed a progressive accumulation until d 16 (75 ng ng^{-1} cDNA).

The fungus inoculation resulted in transient peaks of *STS* and *PMT* transcripts at 5 (200 ng ng^{-1} cDNA) and 9 (130 ng ng^{-1} cDNA) d after beginning of experiment, respectively (Figs. 3 and 4C). Ozone fumigation at 0.15 μL L^{-1} decreased slightly the peak of *STS* transcript accumulation, and delayed the peak of *PMT* transcripts until d 16 (110 ng ng^{-1} cDNA). Ozone fumigation of 0.3 μL L^{-1} prolonged the peak of *STS* to a steady-state level of 150 ng g^{-1} cDNA, whereas *PMT* transcript amount was still increasing 16 d after beginning of experiment at 170 ng g^{-1} cDNA. Fungus increased approximately 2-fold the peak of *STS* and *PMT* transcript amounts occurring in wounding with a 4-d earlier occurrence for *STS*. *STS* and *PMT* transcript amounts exhibited similar responses to ozone. At 0.15 μL L^{-1} a significant decrease occurred, whereas at 0.3 μL L^{-1} the peak level was prolonged or slightly increased.

DISCUSSION

PMT Gene

The genomic sequence matches the open-reading frame of the cDNA previously reported (Chiron et al., 1998), showing only two base changes that did not affect the polypeptide sequence. A possible discrepancy could be observed between the 5′-untranslated

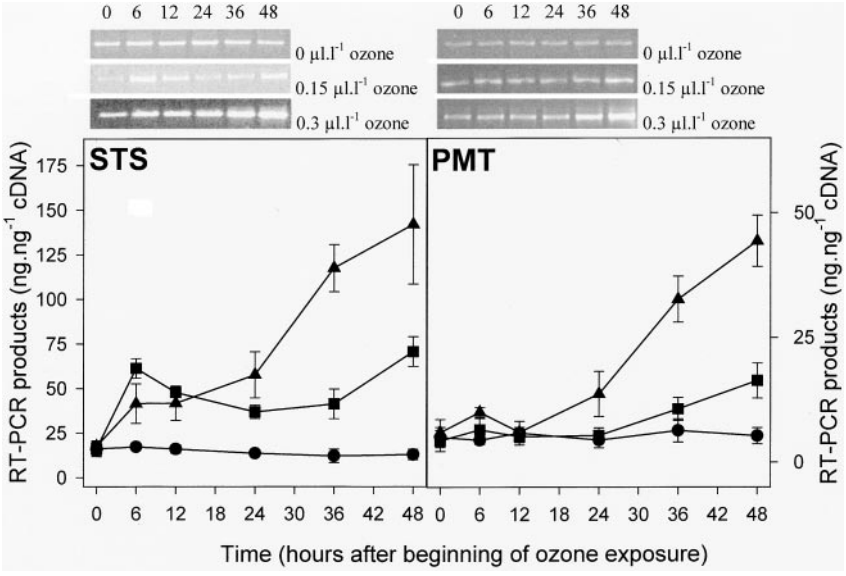

Figure 2. Induction of transcript levels of *STS* and *PMT* by ozone in Scots pine needles. RT-PCR was carried out on 5 μg of RNA isolated from needles at 0, 6, 12, 24, 36, and 48 h after the beginning of ozone treatment (0, 0.15, and 0.3 μL L^{-1} ozone for 10 h per day during 2 d). RNA was isolated according to Kiefer et al. (2000). ●, PCR products in pine needles treated with 0 μL L^{-1} ozone; ■, 0.15 μL L^{-1} ozone; and ▲, 0.3 μL L^{-1} ozone. Bars represent ± SE (n = 3 trees; at least two samples per tree and two RT-PCR reactions per sample).

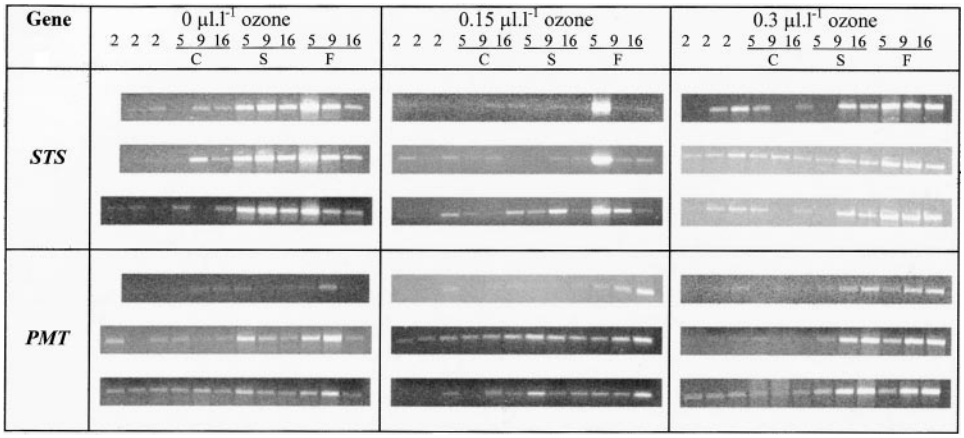

Figure 3. Indcution of mRNA levels of *STS* and *PMT* by ozone and fungus in Scots pine phloem. Pine trees were treated with ozone for 10 h/d, during 2 d. Then inoculations were performed with a 3-week-old culture of *Leptographium wingfieldii* (F) or with sterile malt agar (S). C, Controls without inoculations. RT-PCR was carried out on 5 μg RNA isolated from phloem at 2, 5, 9, and 16 d after onset of ozone fumigation. Ethidium bromide-stained RT-PCR products of three individual saplings are shown for each treatment.

region reported for the cDNA and the genomic leader sequence since the CAP signal ended 10 bp upstream of the 5′-non-coding start of the cDNA. Consequently, the beginning of the cDNA could be 10 bp upstream and the TATA box would be located at −36, which is more consistent with its positions in walnut chalcone synthase (CHS) (−32) (Claudot et al., 1999), parsley caffeoyl-coenzyme A (CoA)-*O*-methyltransferase (CCoAOMT) (−32) (Grimmig and Matern, 1997), grapevine *STS* (−33) (Schubert et al., 1997), and parsley PAL (−30) (Logemann et al., 1995).

The characterization of the promoter allows the determination of potential cis-regulatory elements that are possibly related to the rapid transient accumulation of *PMT* mRNA by treatment of pine with ozone and fungal elicitor. Genes encoding *PAL*, cinnamate 4-hydroxylase, and 4-coumarate-CoA ligase (*4CL*) are well known to be largely controlled at the level of transcription and to be coordinately expressed in response to both developmental and environmental stimuli in many plant species. Members of the *Myb* family are involved in the regulation of these phenylpropanoid genes (Rushton and Somssich, 1998).

MREs were located in Scots pine *PMT* promoter at positions −121, −129, −194, −230, −310, and −358 (Fig. 1). The MREs at positions −358, −310, and −230 have been described in parsley as boxes L, A, and P, respectively for nearly all known *PAL* and *4CL* genes (Logemann et al., 1995) and also in parsley CCoAOMT genes (Grimmig and Matern, 1997). These elements alone, or the promoter region containing all of them together, failed to confer elicitor or light responsiveness of a reporter gene in transient expression assays. Consequently these elements appear to be necessary but not sufficient for elicitor or light-mediated *PAL* and *4CL* gene activation (Logemann et al., 1995). Moreover, no example of a gene outside those involved in general phenylpropanoid metabolism, whose promoter contains a complete set of all three boxes, is reported, further supporting their functional importance in the coordinate regulation of these genes.

A G box located proximal to the TATA box is a widely dispersed sequence motif in eukaryotic promoters. G boxes were present in Scots pine *PMT* promoter at positions −147 and −165 and in an inverse orientation at position −353. Plant G box or its core ACGT motif has been reported to bind different nuclear factors. Functional analysis of plant promoters has demonstrated the role of the G box in promoter activation by various signals including light, abscisic acid, and UV light (Faktor et al., 1996). The conservation of both G box and H box between TATA and G boxes in different *CHS* promoters emphasizes their importance as regulatory motifs (Faktor et al., 1996). One H box is present in Scots pine promoter in an inverse orientation at position −140 between TATA and G boxes. Both G and H boxes were found in the proximal region of the promoters of a number of genes encoding phenylpropanoid biosynthetic enzymes, including PAL, 4CL, and CHS (Zhu et al., 1996). G box and H box located near the TATA box were described to be both essential for floral expression (Faktor et al., 1996). G boxes are involved in the regulation of diverse genes by developmental- and pathogen-derived signals as well as abcisic acid, light, UV irradiation, wounding, as well as pathogen signals. The H box has a much more restricted distribution, being characteristic of phenylpropanoid biosynthetic gene promoters (Zhu et al., 1996). G box and H boxes, in combination, are necessary and apparently sufficient for feed-forward stimulation by 4-coumaric acid (Loake et al., 1992). The H box is also present in the parsley *CHS* and *PAL*

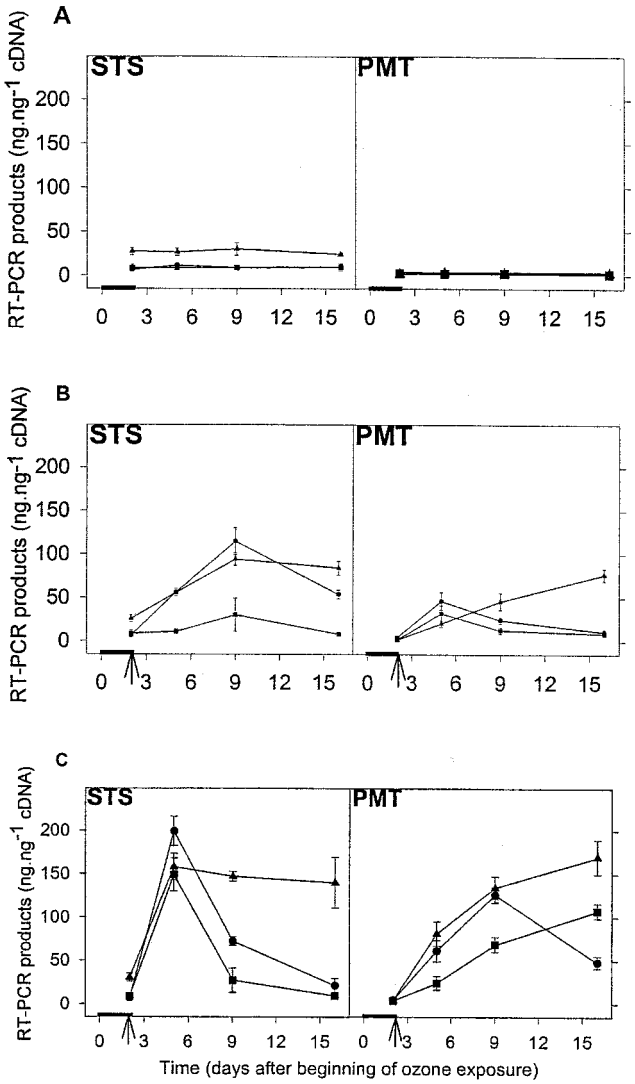

Figure 4. Time course of changes in *STS* and *PMT* mRNA abundance. A, Transcript levels of *STS* and *PMT* in Scots pine phloem. Pine trees were treated with ozone (0.15 and 0.3 μL L^{-1} ozone for 10 h per day during 2 d). B, Induction of transcript levels of *STS* and *PMT* by ozone and wounding in Scots pine phloem. Sterile inoculations were performed at $t = 2$ d with 3-mm discs of sterile malt agar culture (indicated by an arrow). C, Induction of transcript levels of *STS* and *PMT* by ozone and fungus in Scots pine phloem. Pine saplings were fumigated with ozone (0.15 and 0.3 μL L^{-1} ozone for 10 h per day during 2 d). Fungal inoculations were performed at $t =$ 2 d with 3-mm discs of a 3-week-old *L. wingfieldii* culture (indicated by an arrow). RT-PCR was carried out on 5 μg of RNA isolated from phloem at 2, 5, 9, and 16 d after beginning of ozone treatment. RNA was isolated according to Kiefer et al. (2000). ●, PCR products in pine phloem treated with 0 μL L^{-1} ozone; ■, 0.15 μL L^{-1} ozone; and ▲, 0.3 μL L^{-1} ozone. Bars represent ± SE ($n = 3$ trees; see Fig. 2).

promoters, and functional analysis indicates that this cis-element is involved in UV induction (Loake et al., 1992). Enhancer or activator elements dramatically increase the transcriptional activity of certain eukaryotic genes. A copy of the SV40 enhancer core sequence is found at position −356 in the *PMT* promoter. Such enhancer sequences were previously reported from the promoters of *Phaseolus vulgaris PAL* genes (Cramer et al., 1989) and parsley CCoAOMT (Grimmig and Matern, 1997).

Recently, elicitor responsive elements (W boxes) TTGACC have been reported (Raventós et al., 1995; Rushton et al., 1996). Such W boxes are present at position −440 and in an inverse orientation at positions −55, −199, and −263 in the *PMT* promoter (Fig. 1). Elicitor responsive element-like sequences occur in the promoter of different defense-related genes, including *PR1* of parsley (Rushton et al., 1996), in the *CHS* promoter of maize (Franken et al., 1991), and in the *STS* promoter of grapevine (Schubert et al., 1997; Ernst et al., 1999). Elicitor responsive elements may be quite universally responsible for the induction of plant defense pathways.

In the grapevine *STS* promoter, the ozone responsive region (−430 to −280) differs from the pathogen responsive region (−280 to −140) (Schubert et al., 1997; Ernst et al., 1999). In the Scots pine *PMT* promoter, W boxes were more abundant in the region between −263 and −50. Comparison of the ozone responsive *STS* promoter region did not reveal a strong sequence similarity to the *PMT* promoter. This has also been found for a senescence-associated gene promoter in Arabidopsis (Miller et al., 1999). Therefore, the presence of possible ozone responsive elements has still to be proven. Taken together the similarities of motifs found in the pine *PMT* and grapevine *STS* promoters may indicate interactions of several cis-elements in the ozone- and pathogen-induced transcript levels of stilbene biosynthetic genes.

PMT and *STS* Induction by Ozone and Fungal Pathogen

The dose-dependent ozone induction accumulation of *STS* and *PMT* transcripts in needles (Fig. 2) is in good accordance with previously found increases of stilbene contents, STS and PMT enzyme activities, and *STS* transcripts in Scots pine seedlings (Rosemann et al., 1991; Zinser et al., 1998). Comparison of all these data suggests that stilbene metabolites seemed to be regulated at the transcriptional level.

Ozone did not induce *STS* and *PMT* mRNA in phloem, indicating no systemic ozone effect (Figs. 3 and 4A). Similarly, in non-mycorrhizal roots of Scots pine seedlings, no ozone effect on stilbene metabolites was found (Bonello et al., 1993).

Wounding led to transient inductions of *STS* and *PMT* transcripts (Figs. 3 and 4B), which are increased by fungus during the first week after inoculation (Figs. 3 and 4C). The stilbenes PS and PSM were detected in reaction zone only in phloem wounded or inoculated by a bark-beetle associated fungus, in agreement with the proposed stilbene involvement of tree resistance (Lieutier et al., 1996; Bois and Lieutier, 1997). Ozone resulted in transient *STS* and *PMT*

mRNA increases in needles (Fig. 2), thus illustrating similarities between ozone- and pathogen-induced transcript increase.

Combined Stress

The impact of simultaneous environmental stresses on plants is not well known. Both positive and negative interactions seem to exist among different stress factors with regard to gene expression (Örvar et al., 1997; Xiong et al., 1999). When applied before wounding or fungal attack, 0.15 μL L^{-1} ozone decreased pine *STS* and *PMT* transient induction, whereas 0.3 μL L^{-1} ozone restored and prolonged the induction level over 2 weeks (Fig. 4, B and C). As in Scots pine *PMT* is present as a multigene family (Chiron et al., 1998), different members of the family might be differentially regulated upon environmental stimuli. There also could be a competition between induction and degradation of transcripts leading to the steady state level measured, or different levels of stress might affect the balance differently. Nevertheless, this different effect of the two ozone concentrations applied requires further investigations. Similar contrary reactions have been reported for birch clones exposed to ozone and/or drought interactions (Pääkkönen et al., 1998) as well as for *Heterobasidion*-challenged roots of ozone-treated Scots pine seedlings (Bonello et al., 1993). It is interesting that a systemic ozone effect on stilbene metabolites in roots of Scots pine seedlings was found only in pathogen-challenged seedlings (Bonello et al., 1993), similar as found in this report in the phloem of Scots pine saplings. Future experiments should focus on determining such complex interactions of ozone with various abiotic/biotic stress factors.

MATERIALS AND METHODS

Plant and Fungal Material

Seven-year-old Scots pine (*Pinus sylvestris*) saplings were purchased from the Bauchery nursery (Crouy-sur-Cosson, Loir et Cher, France) and were further cultivated for 5 months under a pergola. After the experimental treatments, the trees were transferred back to the pergola, and 1 year later the rate of survival was determined. *L. wingfieldii* was from the Institut National de la Recherche Agronomique collection (Orléans, France), and was initially isolated from the bark beetle *Tomicus piniperda* and its galleries. It was purified by monospore culture and cultivated on malt-agar at 22°C in dark (Lieutier et al., 1989). Cultures were preserved at 4°C with a yearly passage on Scots pine logs at 22°C to retain their activity.

Ozone Treatment

In April 1998, saplings were acclimated for 6 d in the GSF phytotron walk-in chambers (Neuherberg, Germany; Thiel et al., 1996). The light period was 14 h d^{-1} (1,300 μE m^{-2} s^{-1} photosynthetically active radiation; 22.5 W m^{-2} UV-A; 0.45 minimum erythemal dose h^{-1} UV-B); day/night temperatures were 22°C/16°C and day/night relative humidities were 70%/85%. Saplings were fumigated with ozone (0.15 or 0.3 μL L^{-1}) for 10 h per day during 2 d.

Inoculations

Forty-eight hours after the beginning of ozone fumigation, two sterile and two fungal inoculations were carried out onto every tree in each chamber. Calibrated agar discs (3-mm diameter) of a 3-week-old sporulating *L. wingfieldii* culture were introduced into the tree at the cambium level, according to a method described by Wright (1933). Mock inoculations without fungus were performed with 3-mm diameter sterile malt agar discs. Inoculations were made at two different levels of the trunk with a distant of at least 20 cm. A 4 (horizontal) × 7-cm (vertical) rectangle of phloem tissue was removed around each inoculation site and used for RNA analysis after discarding a 1 × 2-cm rectangle of phloem tissue directly enclosing the inoculation point.

Nucleic Acid Isolation

Lyophilized needles (70–90 mg), omitting the current year flush and lyophilized phloem tissue (100 mg), were ground to a fine powder, and total RNA was isolated as described (Kiefer et al., 2000). Genomic DNA was extracted from adult Scots pine needles according to protocol 1 in Csaikl et al. (1998).

PMT Genomic Clone Isolation

The promoter sequence was obtained using gene specific reverse primers designed from the *PMT* cDNA sequence (Chiron et al., 1998), according to the method described by Cormack and Somssich (1997), and 1.5 μg of Scots pine genomic DNA was completely digested with 20 units *Eco*RI. The DNA was precipitated 5 min on ice with 2 volumes of isopropanol, washed with 70% (v/v) ethanol, and resuspended in 15 μL H_2O. After a 5-min incubation at 90°C, the DNA was polyadenylated with 0.5 mM dATP and 1.5 mM $CoCl_2$ in 20 μL of terminal transferase (TdT) buffer (Roche, Mannheim, Germany) containing 50 units of TdT at 37°C for 1.5 h. The reaction was stopped by heating the sample at 72°C for 5 min.

The first PCR was performed with 1/10 volume of the polyadenylated DNA (150 ng), 100 pmol of gene specific primer 1 (5′-TCCCGAGTTCCATGCCCAGAA-3′), 100 pmol of universal-T17 primer (5′-GTAAAACGACGGCCAGTCG-ACTTTTTTTTTTTTTTTTT-3′), 200 μM dNTPs, and 5 units of *AGSGold* DNA polymerase (*AGS*) in 100 μL of 1 × *AGSGold* buffer with thermal cycling conditions consisting of an initial denaturation step at 94°C for 1 min, followed by 35 cycles of 1-min denaturation at 94°C, 1-min primer annealing at 60°C, and 3-min extension at 72°C, with a final 10-min extension period at 72°C.

The second PCR was carried out using 1 μL of the first PCR product, under the same conditions as the first PCR

except that 100 pmol of gene specific primer 2 (5′-GCC-GATCCCATTTCGAATCC-3′) and 100 pmol of universal primer (5′-GTAAAACGACGGCCAGT-3′) were used. The final PCR product was purified and cloned into pGEM-T vector (Promega, Madison, WI) according to the manufacturer's instructions. The plasmids were sequenced commercially (MWG, Ebersberg, Germany).

RT-PCR Analysis

Total RNA (5 μg) from pine needles or phloem tissue was DNaseI digested and reverse transcribed for 1 h at 42°C by 200 units SuperscriptII RT (Life Technologies/Gibco-BRL, Cleveland), with 1× corresponding buffer, 10 mM dithiothreitol, 0.4 mM each dNTP, 100 nM oligo(dT)$_{12–18}$ primer (Life Technologies/Gibco-BRL), and 10 units RNase inhibitor (Life Technologies/Gibco-BRL).

The cDNA was quantified according to a method described by Kiefer et al. (2000) and 10 ng were used for PCR with 2.5 units *Taq* polymerase (Amersham-Pharmacia Biotech, Freiburg, Germany), 1× corresponding buffer, 0.2 mM each dNTP, and 0.2 μM 3′ and 5′ primers. Amplification was performed during 35 cycles of 1-min denaturation at 94°C, 1-min primer annealing at 58°C (PMT and cab primers) or 62°C (STS primers), and 2-min elongation at 72°C. Reaction products were analyzed by electrophoresis through a 1% (w/v) agarose gel, visualized under UV-light after ethidium bromide staining, and quantified using the Picogreen method (Molecular Probes, Leiden, The Netherlands). The authenticity of the PCR products was checked by two directional partial sequencing using the Thermo Sequenase Kit (Amersham-Pharmacia Biotech).

ACKNOWLEDGMENTS

The authors thank Evi Kiefer for helpful assistance, Werner Heller and Dieter Treutter (Technical University of Munich) for excellent cooperation, and Franck Brignolas (University of Orléans) for critical reading of the manuscript. We are grateful to the staff of the GSF phytotron for their excellent technical assistance.

Received March 21, 2000; accepted July 7, 2000.

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