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ORIGINAL PAPER

Ozone affects shikimate pathway genes and secondary metabolites in saplings of European beech (*Fagus sylvatica* L.) grown under greenhouse conditions

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Abstract The shikimate pathway plays a central role in the formation of aromatic intermediates in the production of stilbenes, flavonoids and lignins. Ozone effects on the levels of transcripts in this pathway were studied in saplings of European beech. Complementary DNA (cDNA) clones of all genes of this pathway were isolated, and quantitative real-time RT-PCR (qRT-PCR) using RNA isolated from leaves of ozone-treated saplings showed a strong induction of 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase 1 (*DAHPS1*), *DAHPS3*, 3-dehydroquinate dehydratase/shikimate dehydrogenase (*DHQD/SD*), 5-*enol*pyruvyl-shikimate-3-phosphate synthase (*EPSPS*), and chorismate mutase (*CM*) transcripts. In contrast, *DAHPS2*, 3-dehydroquinate synthase (*DHQS*), shikimate kinase (*SK*), and chorismate synthase (*CS*) transcripts were only weakly induced. Earliest induction could be observed after 2 days of ozone treatment for *DAHPS1*, *SK*, *EPSPS* and *CM*. The coordinated regulation was evident for 3–5 weeks after the onset of ozone fumigation, and increased transcript levels were still detectable after another 7 weeks. Western blot analyses of DAHPS3 and DHQD/SD showed an increased protein level in agreement with the increased transcription levels. Ozone-dependent leaf lesions appeared 7 weeks after onset of ozone exposure. Strongly elevated were levels of conjugates of salicylic (SA) and gentisic acids (GA), either derived directly from chorismate, the key product of the shikimate pathway, or via phenylalanine, cinnamic, and benzoic acids. Concentrations of cell wall-bound phenolic compounds increased in both control and ozone-treated saplings with the latter showing slightly higher levels. Interestingly, however, this increase of cell wall-bound phenolics was accompanied by a decrease of soluble phenolics, which may indicate their deposition into the cell wall.

Keywords *Fagus sylvatica* · Gene expression · Ozone · Phenolic metabolites · Shikimate pathway

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Introduction

Ozone is now accepted as an 'abiotic elicitor' inducing defense pathways in plants (Sandermann et al. 1998). Ozone exposure often results in the accumulation of phenolic secondary metabolites (Langebartels et al. 2002), which requires increased carbon output by primary metabolism. To further examine this hypothesis of stress-induced changes in resource allocation, we decided to analyze the regulation of the shikimate pathway in European beech under the influence of ozone. European beech is the most abundant broadleaf tree in Germany (Schütt et al. 1992). As it is also of major importance for European forest industry, it was chosen as an experimental species for our studies to obtain deeper molecular insights into the possibly detrimental effects of ozone on European broad leaf forest ecosystems.

The shikimate pathway, is based on the primary metabolism, starts from phosphoenolpyruvate and erythrose 4-phosphate, and ends with chorismate. Chorismate is the precursor to a large number of phenolic metabolites, e.g., salicylic acid (SA), flavonoids and lignins. Thus, the shikimate pathway links primary metabolism with a wide range of phenolic secondary plant metabolites. Seven enzymatic reaction steps are known, catalyzed by the enzymes 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (DAHPS, EC 2.5.1.54), 3-dehydroquinate synthase (DHQS, EC 4.2.3.4), the bifunctional enzyme 3-dehydroquinate dehydratase/shikimate dehydrogenase (DHQD/SD, EC 4.2.1.10/EC 1.1.1.25), shikimate kinase (SK) (EC 2.7.1.71), 5-*enol*pyruvylshikimate 3-phosphate synthase (EPSPS, EC 2.5.1.19) and finally chorismate synthase (CS, EC 4.2.3.5). In contrast to bacteria, where these enzymes are highly feedback-regulated, plants contain isoforms differentially regulated at the transcriptional level either during plant development or by environmental stimuli like pathogen attack, wounding, light or fungal elicitors (McCue and Conn 1989; Keith et al. 1991; Henstrand et al. 1992; Muday and Herrmann 1992; Görlach et al. 1995; Bischoff et al. 1996; Batz et al. 1998; Bischoff et al. 2001). Chorismate, the end product of the shikimate pathway, is a central intermediate in various important metabolic pathways leading, for example, to prephenate via chorismate mutase (CM, EC 5.4.99.5), and then to L-tyrosine and L-phenylalanine; or to anthranilate via anthranilate synthase (EC 4.1.3.27) and finally to L-tryptophan and its products; or to isochorismate, itself a central intermediate in the production of a broad range of important plant metabolites. This branch point is therefore an obvious target for complex metabolic feedback regulation.

In the present work, we have cloned at least one isoform of all of the shikimate pathway genes from European beech. With this tool, we analyzed the ozone-induced regulation of these steps on both the gene and protein levels. So far, there are few comprehensive studies on entire shikimate pathway transcripts in herbaceous plants (Bischoff et al. 1996, 2001; Janzik et al. 2005), and this is the first detailed report of a complete shikimate pathway in a tree species. Apart from recently published work on short-term ozone fumigation of tobacco plants (Janzik et al. 2005), little information exists about the ozone regulation of the shikimate pathway genes in plants. An ozone-induced increase in DHQD/SD activity was found in poplar (Cabané et al. 2004), and a study on adult European beech trees gave the first indications that exposure to ozone levels twofold higher than normal could produce slight changes in transcript levels of shikimate pathway genes (Betz et al. 2008). Plants have long been known to exhibit responses to ozone exposure resembling a hypersensitive response following pathogen attack. Ozone has therefore been qualified an abiotic elicitor of plant defense reactions (Sandermann et al. 1998). These responses include phytoalexin production, synthesis of pathogenesis-related (PR) proteins, and formation of various aromatic metabolites like lignins and flavonoids (Langebartels et al. 2002). Many of these metabolites are formed via the phenylpropanoid pathway, and the first enzyme of this pathway, phenylalanine ammonia-lyase, has frequently been shown to be up-regulated at the levels of both transcription and enzymatic activity in herbaceous plants and trees (Eckey-Kaltenbach et al. 1994; Tuomainen et al. 1996; Koch et al. 1998). Extensive early work on the shikimate pathway has shown a complex interplay between primary and secondary metabolism (Batz et al. 1998; Logemann et al. 2000).

SA, an important signaling substance in plants, is derived from the shikimate pathway and is frequently up-regulated in herbaceous plants following pathogen attack (Durner et al. 1997) or ozone exposure (Sharma et al. 1996; Rao and Davis 1999; Rao et al. 2002). SA is a central component in plant defense signaling as part of the so-called systemic acquired resistance response. It is involved in the oxidative burst and in cell death (for review see Métraux and Durner 2004). Far less information is known about SA and ozone in trees. In an ozone-tolerant birch clone, free SA accumulated upon ozone treatment, whereas no SA accumulated in an ozone-sensitive one (Vahala et al. 2003). In an ozone-resistant clone of poplar, however, no significant change was found, while the level of free SA increased in a sensitive clone (Diara et al. 2005). SA may be formed by two different ways from the shikimate pathway, either directly from chorismate via isochorismate, or through a more complex route via phenylalanine, cinnamic and benzoic acid (Wildermuth et al. 2001; Métraux 2002; Shah 2003). We therefore analyzed SA metabolism together with its hydroxylation product, 2,5-dihydroxysalicylic acid [gentisic acid (GA)]. Besides its antioxidant

properties due to its *p*-dihydroxy configuration, GA has antifungal activity and can be a part of the response to systemic infections in plant–pathogen interaction (Bélles et al. 2006). GA in tomato and cucumber shows a different activity than SA for the induction of defense proteins (Bélles et al. 2006). When beech trees are exposed to ozone concentrations twice the ambient level, conjugated GA accumulates together with conjugated SA (Nunn et al. 2005a).

We also studied ozone-dependent changes in phenolic compounds, which are end products linked to the shikimate pathway and have been implicated in the defense against ozone (Rosemann et al. 1991; Heller 1994). Phenolic compounds are known to be involved in plant protection against biotic as well as abiotic stresses like UV radiation or ozone. They may also contribute to communication within and between plants, for example, as a fertility factor in auxin signaling or in allelopathy; between plants and microorganisms, such as in plant–rhizobia interactions; or between plants and animals (for review see Peer and Murphy 2006). Phenolic aglyca are frequently cytotoxic at higher concentrations, even at the site of their own synthesis (Wagner and van Brederode 1996). Therefore, they are usually conjugated and stored in the cellular vacuole (Harborne 1993). Alternatively, they may be exported and integrated into the cell wall or may even be excreted and deposited on the surface of the tissues where they have been formed (Wollenweber et al. 2003, 2005).

Given that radical scavenging activity is another important function of polyphenolic compounds (Bors et al. 1998; Langebartels et al. 2002), it is not surprising that plant exposure to ozone results in an accumulation of phenolic compounds (Sandermann et al. 1998). In parsley, furano-coumarins and flavone *O*-malonylglycosides were detected in ozone-treated parsley plants (Eckey-Kaltenbach et al. 1994). Similarly, ozone induction of quercetin and kaempferol derivatives has been observed in soybean plants. This response is accompanied by a decrease in hydroxycinnamic acid derivatives. However, it has not been shown whether these metabolites are degraded, deposited into the cell wall, or used as substrates in further metabolic steps (Biolley et al. 1998). In trees, ozone-dependent induction of phenolic metabolites has already been described (Langebartels et al. 1997). Exposure to ozone levels two times the normal amount did not produce any significant effects either in the sun or shade leaves of adult European beech in a forest stand (Bahnweg et al. 2005). However, ozone treatment of beech saplings a few years old resulted in up- or down-regulation of flavonoid glycosides, caffeic acid derivatives and a biphenyl compound, particularly in lammas shoots (Zielke and Sonnenbichler 1990; Zielke 1994).

Although physiological responses of forest trees to ozone have been well documented, studies on a molecular biological level have not often be reported. Therefore, we set-out to examine the effects of elevated ozone on the shikimate pathway in European beech in more detail and to analyze transcriptional changes of all genes involved in this pathway in order to understand the effects of ozone on these plants. To complete our understanding of how ozone triggers changes in both primary and secondary metabolism, we analyzed signaling molecules and phenolic compounds linked to the shikimate pathway.

Materials and methods

Plant material and growth conditions

Three-year-old European beech saplings (*Fagus sylvatica* L., provenance 81024; Schlegel Baumschulen, Riedlingen, Germany) were planted in 14-l pots filled with natural forest soil (site Höglwald, Bavaria, Germany; Grams et al. 2002). Saplings were maintained during winter in the institute nursery. Before bud burst, the plants were treated with Promanal® (Neudorff, Emmerthal, Germany), based on rape oil, to prevent insect infestation. In April 2004 the 4-year-old saplings (mean height 100 cm) were transferred to a climate-controlled greenhouse covered with UV-permeable glass sheets to allow photobiological processes comparable to those occurring in the open air (http://www.helmholtz-muenchen.de/eus/index.php; Betz 2005; Olbrich et al. 2005). The saplings were cultivated until full leaf development and were kept at a relative humidity of 70–80% and temperatures of 22–25°C during day time (from 6:00 a.m. to 8:00 p.m.) and 17–20°C during the night (from 8:00 p.m. to 6:00 a.m.). At the beginning of June 2004 saplings were exposed to ozone for 8 h day^{-1} (150 nl l^{-1}) or subambient (<15 nl l^{-1}) ozone concentrations. After 13 days the ozone concentration was increased to 190 nl l^{-1} for further 70 days. We used this relatively high ozone concentrations, as treatment of beech saplings with twice ambient ozone in a phytotron resulted in higher percentage of leaf injury only at the end of the first, respectively, second year of fumigation (Nunn et al. 2005b) Ozone was produced from pure oxygen (Fischer Ozon Generator 500, Neckenheim, Germany) and continuously monitored using a computer-controlled system and a UV-type ozone analyser CSI 3100 (Columbia Scientific Industries, USA). To avoid cabinet effects, the position of the plants was altered weekly. Pest control against beech scale was carried out weekly by hand to spare beneficial insects like larvae of lacewings and hover flies. Thrips were fought off with predatory mites and predatory bugs (Sautter & Stepper, Ammerbuch, Germany). Two to three leaves

were collected for RNA and metabolite analyses from four ozone-treated and four control saplings at different time points from the onset of ozone treatment. They were immediately frozen in liquid N_2 and stored at −80°C until further study.

Isolation of RNA and cDNA transcription

Total RNA was isolated according to Kiefer et al. (2000), treated with RQ1 DNase (Promega, Mannheim, Germany) and quantified photometrically (NanoDrop system; Kisker, Steinfurt, Germany). For cDNA synthesis 5 µg of total RNA (14.5 µl), 1 µl of dNTP (10 mM) and 1 µl of oligo-$dT_{(15)}$ primer (5 µg μl^{-1}) was used for first-strand cDNA synthesis. Reverse transcription was carried out for 4 h at 42°C using superscript II reverse transcriptase, according to the manufacturer's instructions (Invitrogen, Karlsruhe, Germany).

Cloning of homologous beech cDNAs for all shikimate pathway enzymes

To obtain putative shikimate pathway cDNA sequences from beech, degenerated primers were designed based on conserved coding regions identified from a multiple sequence alignment from different plants using MULTALIN (Corpet 1988); Supplementary material Table S1). For each gene, eight identical PCR reactions (25 µl) were prepared containing 2.5 µl reaction buffer (10×), 1 µl $MgCl_2$ (50 mM), 0.5 µl dNTP (10 mM), 0.5 µl of each degenerated primer (10 µM), 0.2 µl cDNA, and 0.1 µl *Taq* polymerase (Invitrogen). In the subsequent PCR reaction (1 × 94°C, 3 min; 35×: 94°C, 30 s; 45–60°C, 50 s; 72°C, 1 min), a temperature gradient was applied to optimize primer annealing. PCR products were cloned into pGEM-T Easy (Promega) and sequenced at a local facility (ABI3100, Applied Biosystems, Darmstadt, Germany).

In order to obtain full-length coding regions of putative shikimate pathway genes from beech, 3′- and 5′-RACE were performed using a GeneRacer-Kit (Invitrogen) according to the manufacturer's protocol with gene-specific primers derived from the sequence data of the cloned cDNA fragments. The resulting full-length cDNA sequences were published under NCBI accession numbers DQ166519-DQ166527 and AY705445.

Quantitative real-time RT-PCR (qRT-PCR)

qRT-PCR was performed in a volume of 12.5 µl of SYBR Green ROX kit (ABgene, Hamburg, Germany), 0.5 µl gene-specific forward primer (10 µM), 0.5 µl gene-specific reverse primer (10 µM) (Supplementary material Table S2), 0.5 µl cDNA and 11 µl H_2O, using an ABIPrism 7700 Sequence Detector (Applied Biosystems, Darmstadt, Germany). The PCR conditions were as follows: 1 cycle at 50°C for 2 min and at 95°C for 10 min; 40 cycles at 95°C for 15 s and at 60°C for 1 min. Fluorescence signals were analyzed using the 7500 System Software (Applied Biosystems). As an internal reference, 26S rRNA was used, and the relative expression ratio (R) was calculated according to the "Delta–delta" method described by Applied Biosystems.

$$R = \frac{2^{\Delta Ct\,\text{target(control}-\text{sample)}}}{2^{\Delta Ct\,\text{reference(control}-\text{sample)}}}$$

Protein analyses

Protein expression of DAHPS3 and DHQD/SD, production of monoclonal antibodies specific for DHQD/SD and DAHPS3 is described in the Supplementary material Text S1.

Extraction and quantification of total soluble protein from beech leaves

A measure of 100 mg freshly ground leaf material was mixed with 1 ml of extraction buffer [200 mM boric acid pH 8.8, 2 mM EDTA, 0.5 mM EGTA, 1.5% PVPP, 5 mM DTT, 1 tablet of complete mini protease inhibitor (Roche, Mannheim, Germany) per 10 ml extraction buffer] (Liegl 1999). After a short sonication step (10 s; Sonifer Cell Disrupter B15, Branson, Dunbury, USA) the extract was centrifuged (3 × 20,000*g*, 4°C). Protein quantitation was performed in triplicate according to Bradford (1976) using a ThermoMax microplate reader (Molecular Devices, München, Germany). To prepare a standard curve, a dilution series of BSA (100–1,000 µg ml^{-1}) was used.

Western blot analysis

For all time points analyzed, 50 µg of raw protein extract was loaded on a SDS-polyacrylamide gel (10%). Protein separation was carried out using a MightySmall vertical gel electrophoresis device (Amersham, Freiburg, Germany) at 4°C for 70 min and a constant voltage of 250 V.

Proteins were then transferred to a PVDF membrane (Amersham) at 14 V for 45 min with a TransBlot SD semidry blotting system (Biorad, München, Germany). All incubation steps during Western blot analysis were performed under soft agitation (40 rpm) at room temperature in a volume of 20 ml. After blotting, the membrane was equilibrated in TBST-buffer [50 mM Tris, 137 mM NaCl, 0,1% (v/v) Tween 20, pH 7.6] for 10 min, blocked with TBST-MP buffer [TBST including 5% (w/v) fat-free milk powder] for 1 h, washed twice for 15 min with TBST,

incubated overnight in diluted primary antibody solution (1:200 dilution), washed twice for 15 min with TBST, incubated with diluted secondary antibody dilution (peroxidase-conjugated goat-anti-rat IgG + IgM, dilution 1:2,000) and finally washed once with TBST and twice with TBS for 10 min. Detection was performed by incubation in 10 ml DAB-solution [1.7 mM 3,3′-diaminobenzidine tetra-hydrochloride in 50 mM Tris buffer (pH 7.6), 0.05% (w/v) hydrogen peroxide].

The membrane was then washed with distilled water in order to stop the detection reaction and finally scanned with an HP scanjet 3970 (Hewlett Packard, Dornach, Germany). Signals were quantified using a gel documentation program (GelScan V5.1, BioSciTec, Frankfurt/Main, Germany).

Determination of phenolic metabolites

HPLC separation of soluble, as well as by alkali treatment liberated, cell wall-bound phenolics was performed according to Turunen et al. (1999) using a 250 × 4.5 mm Spherisorb ODS column (NC, 5 µm, Bischoff, Leonberg, Germany) at 20°C and a flow rate of 1 ml min^{-1}. Soluble phenolic extract (75 µl) was mixed with 20 µl of water and centrifuged for 10 min at 9,000*g*. Samples (injection volume 10 µl) were eluted from the column with a solvent gradient consisting of solvent A [980 ml H_2O + 20 ml 5% ammonium formate in formic acid (98%, w/v)] and solvent B [882 ml methanol + 96 ml H_2O + 20 ml ammonium formate in formic acid (98%, w/v)]. The gradient was as follows: 0–5 min, 100% A (isocratic); 5–55 min, 50% B (linear); 55–85 min, 100% B (linear); 85–90 min, 100% B (isocratic); 90–91 min, 100% A. Cell wall hydrolysates were centrifuged for 10 min at 9,000*g*. Samples (injection volume 20 µl) were eluted from the column as follows: 0–5 min, 100% A (isocratic); 5–45 min, 100% B (linear); 45–50 min, 100% B (isocratic); 50–55 min, 100% A. Detection was at 280 nm with a UV/visible diode-array detector 168 (Beckman Coulter, Unterschleißheim, Germany) and with a fluorescence detector RF10A XL (Shimadzu, Duisburg, Germany).

Determination of SA and gentisic acid GA

Salicylic acid and GA were quantified using a procedure similar to that prescribed by Meuwly and Métraux (1993). Homogenized deep frozen leaf material (100 mg) was extracted with 5 ml methanol and then centrifuged (10 min, 30,000*g*, 4°C). The pellet was re-extracted with 4 ml methanol and then centrifuged. The combined liquid phases were evaporated at 38°C using a rotary evaporator RE111 (Büchi, Flawil, Switzerland). The residue was treated with 2 ml formic acid (20%, v/v) containing 50 µl HCl (32%, v/v) and then extracted subsequently with 5 and 3 ml ethyl acetate/cyclohexane (1:1, v/v). The combined organic phases containing free SA and GA were evaporated as described above. For the analysis of conjugated SA and GA, the water phase was mixed with 1.3 ml HCl (32%, w/v), incubated in Pyrex vials at 80°C for 1 h, then extracted with ethyl acetate/cyclohexane as described above.

Each evaporated sample was re-suspended in 500 µl loading buffer [solvent A (27 mM sodium acetate, 30 mM citric acid, pH 5.0) + 20% methanol, v/v)] and centrifuged for 10 min at 20,000*g* (4°C). HPLC separation of SA and GA was performed on a Merck HPLC system (Intelligent Pump 1 6200A, Merck, Darmstadt, Germany) using RP-C18 Nucleosil column (150 × 4.6 mm, 5 µm, 120A, Bischoff) at a flow rate of 1.5 ml min^{-1}. Samples (injection volume 20 µl) were eluted from the column using a solvent gradient consisting of solvent A and solvent B [27 mM sodium acetate, 30 mM citric acid, 95% (v/v) methanol]. The gradient was as follows: 0–20 min, 100% A (isocratic); 20–25 min, 100% B (linear); 25–35 min, 100% B (isocratic); 35–40 min, 100% A (linear); 40–45 min, 100% A (isocratic). Detection was with a fluorescence detector RF-535 (Shimadzu) and chromato integrator D2500 (Merck) at an excitation of 305 nm and an emission wavelength of 407 nm. The retention time was approximately 5 min for GA and 11 min for SA. These substances were quantitated as follows:

$$\text{free SA(GA)}\left[\text{nmol g fw}^{-1}\right] = k \times \frac{\text{Sample peak [peak unit]}}{\text{SA standard peak [peak unit]} \times \text{fresh weight [g]}}$$

k = 2.41 nmol for SA (GA) and 2.586 nmol for conjugated SA (GA)

Results

Ozone-induced leaf injury

Treatment of beech saplings with ozone (150–190 nl l^{-1}) led to visible leaf symptoms possibly due to cell death (Fig. 1). This ozone-dependent injury occurred at the upper surface, and lesions increased upon increasing exposure time of 42, 49, 55, 63 d after the onset of fumigation (Fig. 1). At the end of the experiment, ozone-treated trees started to shed leaves. Nine out of the 12 treated trees developed severe damage, whereas control trees did not show any symptoms.

Isolation of shikimate pathway genes

We performed a multiple sequence alignment using the MULTIALIN software to design primers for the amplification of shikimate pathway genes (Supplementary

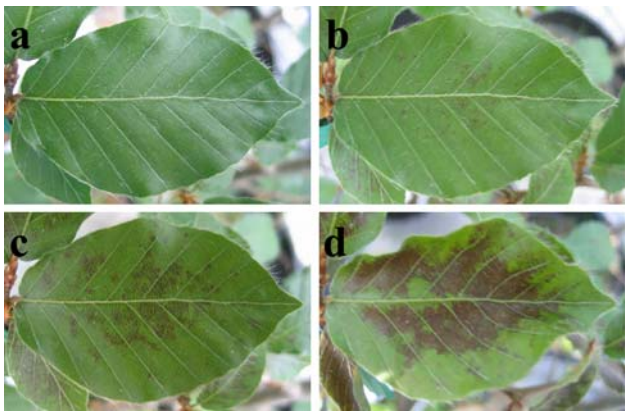

Fig. 1 Deveopment of ozone-induced leaf lesions in leaves of 4-year-old European beech saplings, treated with ozone (150–190 nl l^{-1}, 8 h day^{-1}, 83 days). **a** 42 days, **b** 49 days, **c** 55 days, **d** 63 days

material Table S1). BLASTN analyses of public databases resulted in the identification of all shikimate pathway genes in beech (Table 1, Supplementary material Table S3). With the exception of *DAHPS*, these are the first full-length cDNA clones of shikimate genes from trees. Comparison of the *DAHPS* sequence with three *DAHPS* isoforms from *Arabidopsis thaliana* allowed the construction of degenerate primers that led to the isolation of three isoforms of *DAHPS* from beech. Using the CLUSTALW software and known DNA sequences from *A. thaliana,* all three isolated isoforms were classified; pairwise identities at the DNA and protein level to *A. thaliana* were between 77 and 92%. Data are given in Table 1. The strongest DNA match for fragment B was to *DAHPS2* with 81% identity, whereas fragments A and C showed only 78 and 77% identity, respectively. Therefore, fragment B was named *DAHPS2*. Fragments A and C showed equal identities of 79% with *DAHPS3*. However, at the level of protein sequence, fragment C showed 92% identity, compared to 87% identity with fragment A. According to these results the cloned cDNA fragment A was named *DAHPS1* and fragment C, *DAHPS3*. Running the program "InterProScan" (Zdbonov and Apweiler 2001), the functional domain of the encoded shikimate proteins could be identified. The program "TargetP" (Nielsen et al. 1997; Emanuelsson et al. 2000) was successfully used to predict the amino acid sequence of chloroplast transit peptides, with the exception of DHQD/SD, which was not identified probably because of the very short 5′-region of the gene.

Ozone-dependent expression of shikimate pathway genes

Specific primers for each shikimate pathway gene were successfully designed (Supplementary material Table S2). They were used to analyze ozone-dependent expression by qRT-PCR and were grouped into two classes with expression levels greater than 4.5 and smaller than 3.0 (Table 2). A small, however, significant transcript increase within 2 days was observed for *DAHPS1, SK, EPSPS* and *CM* (Supplementary material Table S4). Comparison of the three isoforms of *DAHPS* clearly showed that only *DAHPS1* and *DAHPS3* were regulated by ozone, whereas *DAHPS2* was only weakly induced and only at the end of the fumigation period (Fig. 2). *DAHPS1* started again to increase at the beginning of visible, macroscopic leaf injury and reached a relative transcription rate of about 30 at the end of the ozone exposure on day 83. *DAHPS3* levels increased when the ozone concentration was adjusted from 150 to 190 nl l^{-1}, and it remained significantly up-regulated until the end of the fumigation period by a factor of 11 (Fig. 2). This differential regulation indicates that the three *DAHPS* isoforms were successfully isolated. The protein expression profile of *DAHPS3* was in agreement with the up-regulation of *DAHPS3* transcription. Western blotting with monoclonal antibodies raised against a heterologously expressed DAHPS protein was used to analyze this amount of DAHPS3 enzyme. In protein extracts of ozone-treated saplings, a single band with increasing intensity at 55, 69, and 83 days after the onset of fumigation consistently appeared between the molecular weight standards of 83 and 175 kDa (Fig. 3). In control saplings,

Table 1 Classification of the putative isoforms of DAHPS of European beech

Clone	Published isoforms of DAHPS from *Arabidopsis thaliana*					
	DNA sequence			Protein sequence		
	DAHPS1 M74819	*DAHPS2* M74820	*DAHPS*3 NM_102090	DAHPS1 M74819	DAHPS2 M74820	DAHPS3 NM_102090
A	**77**	78	79	**83**	83	87
B	76	**81**	78	83	**89**	89
C	76	77	**79**	85	89	**92**

Values correspond to the percentage of pairwise identity to *Arabidopsis thaliana* using the programme CLUSTALW. Bold entries indicate the assignment of the cloned fragments to the corresponding DAHPS isoform

Table 2 Relative abundance of shikimate pathway transcripts in response to ozone

Gene	Expression level > 4.5	SEM	days	Gene	Expression level < 3.0	SEM	days
DAHPS1	32.88	10.14	83	*DAHPS2*	1.67	0.22	76
DAHPS3	11.16	0.97	83	*DHQS*	2.83	0.59	76
DHQD/SD	7.13	1.07	55	*SK*	2.49	0.44	62
EPSPS	4.52	0.92	76	*CS*	2.17	0.19	83
CM	5.07	0.31	55				

Indicated is the highest relative gene expression level

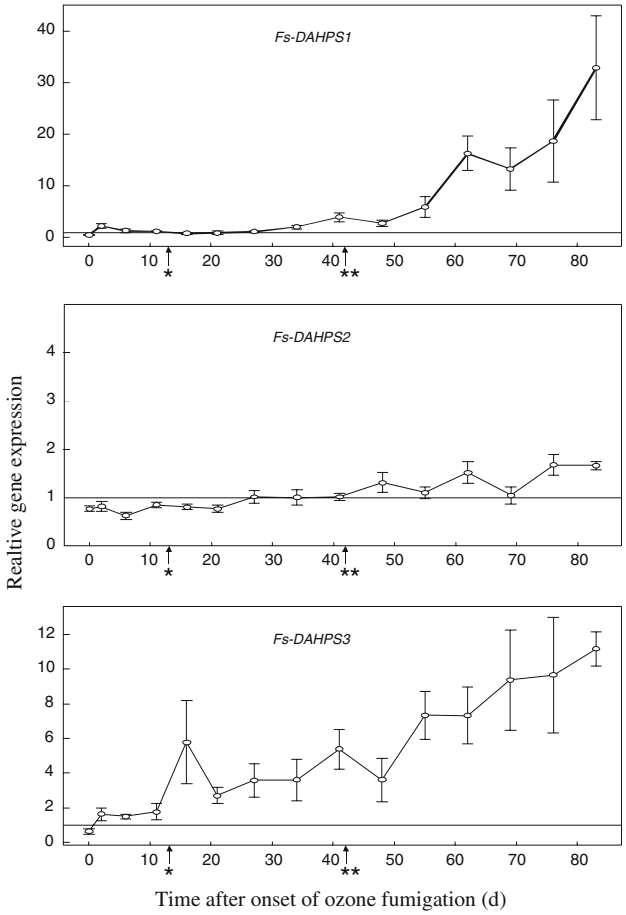

Fig. 2 qRT-PCR analysis of *DAHPS* gene transcripts of total RNA isolated from ozone-treated leaves of European beech. Four-year-old European beech saplings were fumigated with ozone (150–190 nl l^{-1}, 8 h day^{-1}, 83 days); $n = 4$, ±SEM; *single asterisk* indicates increasing ozone concentration from 150 to 190 nl l^{-1}; *double asterisks* indicate first ozone-induced leaf lesions

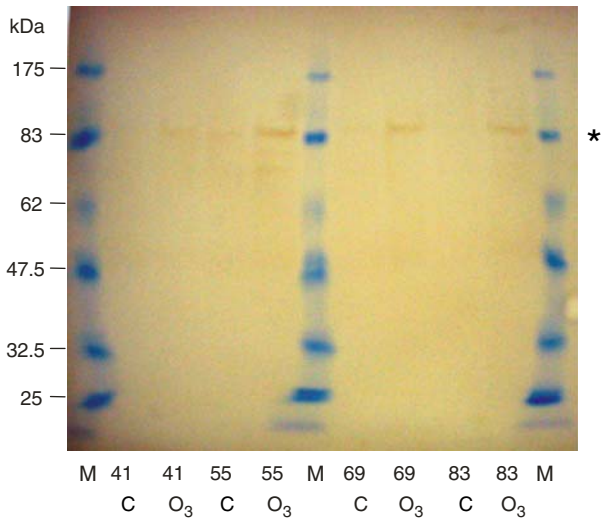

Fig. 3 Western blot analysis. Accumulation of DAHPS3 protein in leaves of 4-year-old European beech saplings (150–190 nl l^{-1}, 8 h day^{-1}, 83 days). DAHPS3-specific hybridization of PVDF membranes containing each 50 µg protein extract of ozone-treated (O_3) and control (C) leaves; *asterisk* indicates the DAHPS3 homodimer protein band

no such signal was visible. However, this band did not fit the expected size of 53 kDa. Possibly, the protein occurred as a homodimer under the conditions used (Herrmann 1995), and it is known that oligomers often can be separated in SDS-polyacrylamide gels under mild conditions (Knight and Fahey 1981; Spelbrink et al. 2005).

DHQS and *SK* were induced only by a factor of two at the end of the experiment (Fig. 4). In contrast, *DHQD/SD* was more clearly up-regulated, significantly from day 16 onwards, and level of transcription increased continuously throughout the ozone treatment (Supplementary material Table S4). Quantitation of the DHQD/SD protein on Western blots with monoclonal antibodies was consistent with the transcript data. Comparing ozone-treated and control leaves, a protein band could be detected after 62 and 76 days of ozone exposure (Fig. 5). A weight of approximately 60 kDa corresponds to the predicted size of DHQD/SD. *EPSPS* showed the highest induction rate of all the genes analyzed in this study after only 2 days of ozone exposure (Fig. 6). Increasing the ozone concentration resulted in a further enhancement of transcription to 4.5-fold higher than baseline. The transcription profile of *EPSPS* over time was very similar to that of *DAHPS3*, although at lower induction factors. *CS* was only slightly induced over time with an increased level at the appearance of macroscopic visible symptoms, similar to *DHQS* and *SK* (Figs. 4, 6). *CM* up-regulation was comparable to the regulation of *DHQD/SD* with the highest relative

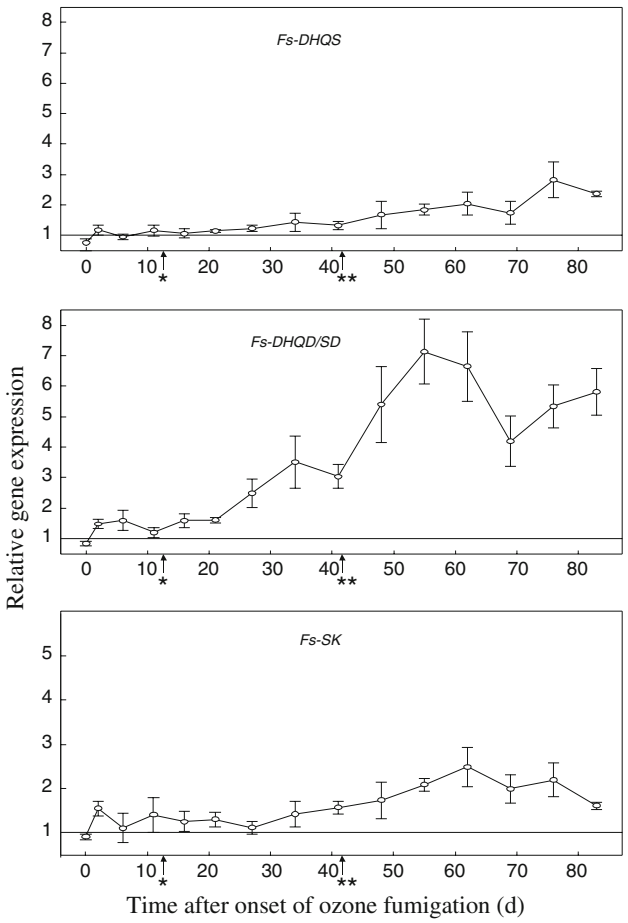

Fig. 4 qRT-PCR analysis of *DHQS*, *DHQD/SD* and *SK* gene transcripts of total RNA isolated from ozone-treated leaves of European beech. Four-year-old European beech saplings were fumigated with ozone (150–190 nl l^{-1}, 8 h day^{-1}, 83 days); $n = 4$ saplings, ±SEM; *single asterisk* indicates increasing ozone concentration from 150 to 190 nl l^{-1}; *double asterisks* indicate first ozone-induced leaf lesions

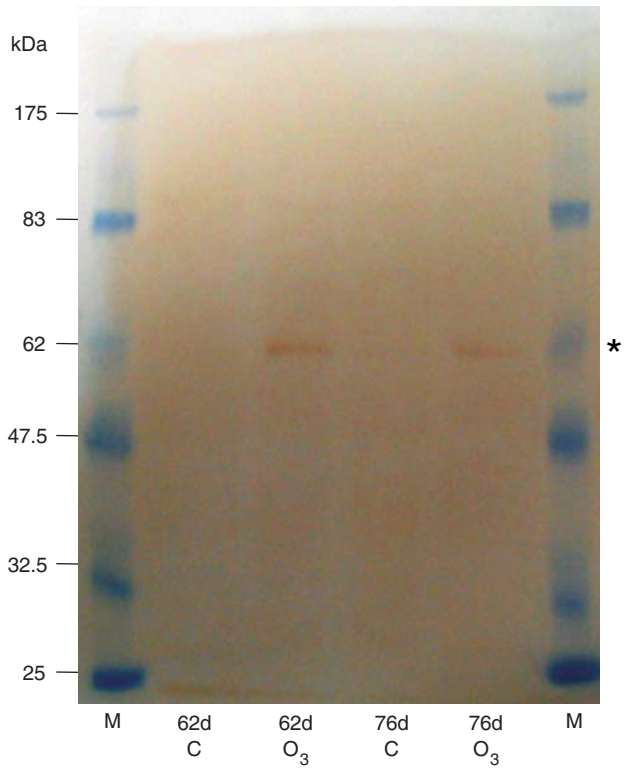

Fig. 5 Western blot analysis. Accumulation of DHQD/SD protein in leaves of 4-year-old European beech saplings (150–190 nl l^{-1}, 8 h day^{-1}, 83 days). DHQD/SD-specific hybridization of PVDF membranes containing each 50 µg protein extract of ozone-treated (O_3) and control (C) leaves; *asterisk* indicates the DHQD/SD protein band

transcription level of about 5 by the end of the fumigation period (Figs. 4, 6).

Ozone-dependent accumulation of SA and GA, and changes in phenolic metabolite levels

Conjugated SA and conjugated GA significantly increased upon ozone treatment, whereas control plants showed clearly lower amounts over the entire exposure period (Fig. 7). No ozone effect, however, was detectable with free SA, the levels of which remained below 0.90 nmol g^{-1} fw independent of the treatment (Fig. 7). Similarly, no free GA was detected under either control or exposure conditions. Conjugated SA increased in ozone-treated leaves from about 14 to 47 nmol g^{-1} fw, and conjugated GA from 111 to 450 nmol g^{-1} fw (Fig. 7).

Most polyphenolic metabolites detected were formed via the shikimate pathway. Total soluble leaf phenolics strongly decreased during leaf development, independently of ozone treatment (Fig. 8a). HPLC analysis of methanolic leaf extracts allowed the differentiation of at least 15 metabolites according to their retention times and diode-array spectra. Several flavan, hydroxycinnamic acid, and flavonol derivatives, as well as 3,3′,4,4′-tetramethoxybiphenyl were characterized. Catechin, a derivative of caffeic acid and one of kaempferol, as well as the biphenyl, showed elevated levels in leaves of ozone-treated beech trees. On the other hand, levels of chlorogenic acid and a ferulic acid derivative were slightly reduced. At the end of the ozone exposure no clear difference between samples of control and treated trees was detectable.

Seven cell wall-bound phenolics that could be released by alkaline hydrolysis were identified. While caffeic acid was reduced under the influence of ozone, the levels of *cis*/*trans* coumaric acid and two kaempferol-3-*O*-glycosides were slightly elevated. As in the soluble metabolite fraction, no difference between control and treated samples was obvious at the end of the fumigation period. Total cell

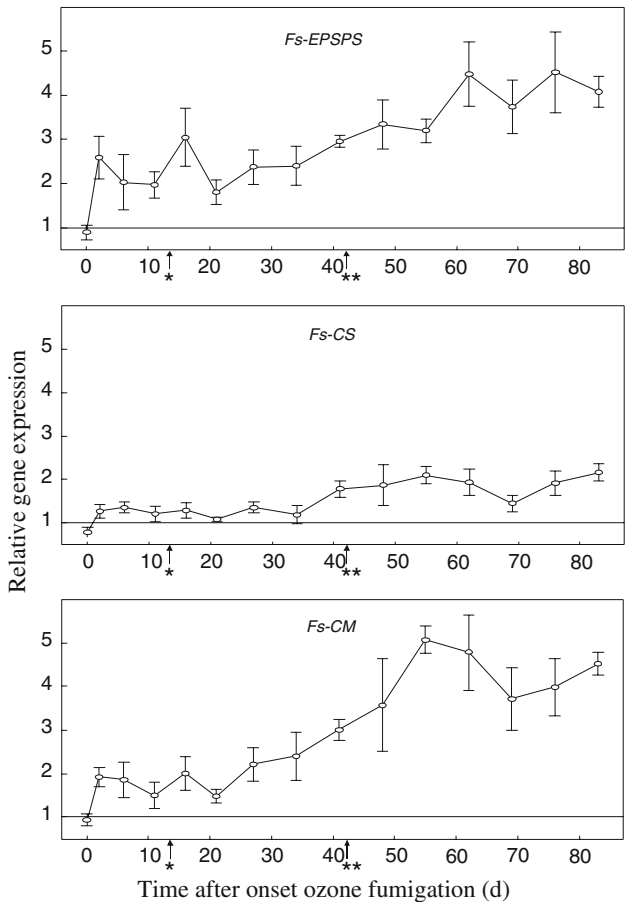

Fig. 6 qRT-PCR analysis of *EPSPS*, *CS* and *CM* gene transcripts of total RNA isolated from ozone-treated leaves of European beech. Four-year-old European beech saplings were fumigated with ozone (150–190 nl l^{-1}, 8 h day^{-1}, 83 days); $n = 4$ saplings, ±SEM; *single asterisk* indicates increasing ozone concentration from 150 to 190 nl l^{-1}; *double asterisks* indicate first ozone-induced leaf lesions

wall-bound phenolics increased from early to late sampling times, with ozone-treated trees showing slightly higher, but still not significant levels (Fig. 8b).

Discussion

Ozone-dependent regulation of the shikimate pathway

Stress-related gene expression of all shikimate pathway genes has so far been extensively analyzed in the Solanaceae family over period no longer than 3 days (Görlach et al. 1995; Bischoff et al. 1996; Janzik et al. 2005). The results of the present 83-day study with European beech showed an extensive reprogramming of the shikimate pathway, which links primary and secondary metabolism. Reprogramming of both primary and secondary metabolism has earlier been shown in parsley cell cultures upon fungal elicitation and UV irradiation (Batz et al. 1998; Logemann et al. 2000). In these studies, stress-dependence of *DAHPS* and *CM* transcription has also been demonstrated. Recently ozone (160 nl l^{-1}; 35 h) was shown to dramatically affect the regulation of the shikimate pathway in the ozone-sensitive tobacco cultivar Bel W3 (Janzik et al. 2005), whereas beech saplings treated with 300 nl l^{-1} for 7–30 days showed only weak transcriptional induction of several shikimate pathway genes (Olbrich et al. 2005; Betz et al. 2008). Similarly adult beech trees, which were treated with twice ambient ozone showed no significant transcriptional differences of shikimate pathway genes (Jehnes et al. 2007; Betz et al. 2008).

In the present study, we have extended the observation period of ozone-induced changes in shikimate pathway transcription to several months. We therefore believe that a direct comparison of our results with published data is difficult, as (1) we studied the experimental plants over a period of 83 days, whereas other studies were run for no longer than 3 days, and (2) most studies were performed with herbaceous plants, and often with suspension cultures. Interestingly four transcripts (*DAHPS1, SK, EPSPS* and *CM*) were significantly up-regulated after 2 days, indicating a rapid response of European beech towards increased

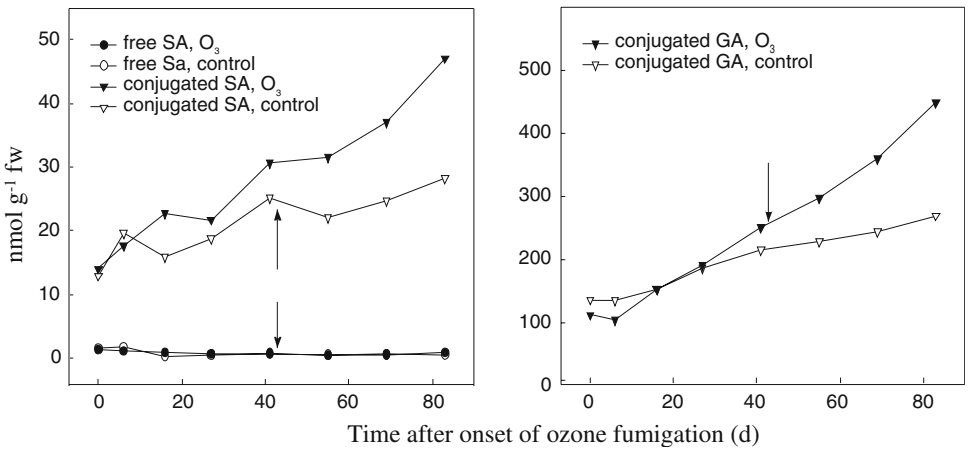

Fig. 7 Accumulation of free and conjugated SA (*left*) and GA (*right*) in leaves of control and ozone-treated 4-year-old European beech saplings (150–190 nl l^{-1}, 8 h day^{-1}, 83 days); $n = 9$ saplings; *arrows* indicate first ozone-induced leaf lesions

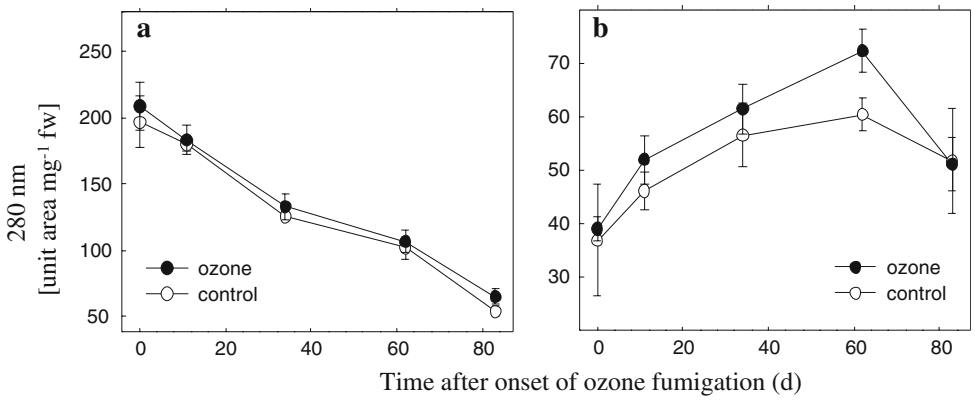

Fig. 8 Total amount of soluble (**a**) and cell wall-bound (**b**) phenolic metabolites in leaves of 4-year-old European beech saplings, exposed to control conditions or ozone (150–190 nl l^{-1}, 8 h day^{-1}, 83 days); $n = 4$ saplings, ±SEM

ozone concentrations (Supplementary material Table S4). Transcripts of the shikimate pathway showed a weak induction after several weeks of ozone exposure, which is in good agreement with a preliminary report on beech saplings using 300 nl l^{-1} (Betz et al. 2008). Induction became much stronger after this initial period (Figs. 2, 4, 6). A strong up-regulation of *DAHPS1* and *DAHPS3* was first observed 40 days after onset of ozone exposure, in parallel with the increase in visible leaf injuries. In contrast, *DAHPS2* transcription was nearly unaffected at that time. A differential induction of several *DAHPS* isoforms upon pathogen attack or elicitation has also been reported for *A. thaliana* and tomato (Keith et al. 1991; Görlach et al. 1995). Wounding of *A. thaliana* resulted in an increased level of *DAHPS1* but not of *DAHPS2* (Keith et al. 1991). In tobacco plants only *DAHPS1* was induced by ozone, while *DAHPS2* and *DAHPS3* were unaffected (Janzik et al. 2005). This difference between beech and tobacco might be explained by the different plant species used, but also by the experimental conditions, as the tobacco studies involved only a short ozone pulse, whereas our study involved ozone fumigation lasting for 83 days. On the other hand, most database entries are given as putative and the discrepancies may reflect misassignments. A comparison of all C_t values of non ozone-exposed trees showed that the constitutively expressed DAHPS2 was the most abundant isoform (Fig. 9). This indicates that, independently of environmental factors, a certain amount of carbon is constantly directed towards chorismate. *DAHPS3* was affected by ozone to the greatest extent, while strong expression of *DAHPS1* correlated to leaf lesion formation. Interestingly 3,3′,4,4′-tetramethoxybiphenyl, a stress-metabolite of beech, occurs exclusively in damaged leaf parts following infection with *Apiognomomia errabunda* (Bahnweg et al. 2005). Thus, an increased *DAHPS1* level may be involved in the formation of this metabolite, although no biosynthetic pathway for this highly symmetrical metabolite has so far been suggested. The protein expression profile for

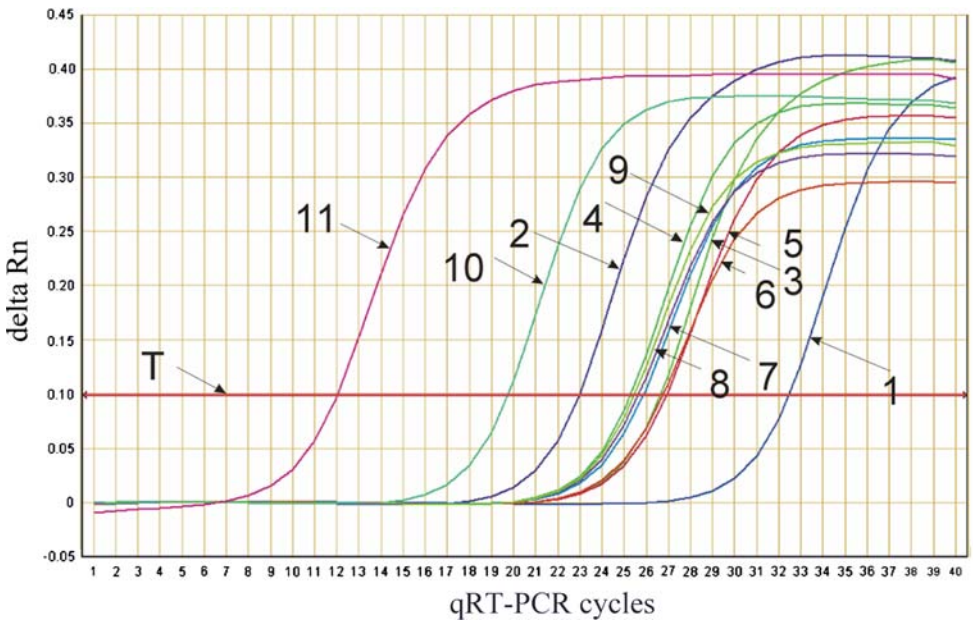

Fig. 9 qRT-PCR expression (*graphically schema*) of putative shikimate pathway genes in leaves of European beech (control sapling, 69 days after start of the experiment). The C_t values are deduced from the intersection of the sigmoidal curves with the threshold value T. *DAHPS1* (1), *DAHPS2* (2), *DAHPS3* (3), *DHQS* (4), *DHQD/SD* (5), *SK* (6), *EPSPS* (7), *CS* (8), *CM* (9), *PR1* (10) and *rbcL* (11); Rn = normalized intensity of the fluorescence

DAHPS3 correlated with its transcript levels (Figs. 2, 3). This indicates a transcriptional control of *DAHPS3*, in agreement with a modulation of overall DAHPS activity by differential isoform expression (Herrmann and Weaver 1999).

Limited information exists about stress-induced regulation of *DHQS* in higher plants. Elicitation of tomato cell cultures resulted in nine-fold transcriptional induction (Bischoff et al. 1996), whereas a down-regulation followed by a weak induction was observed in ozone-treated tobacco (Janzik et al. 2005). In our study with beech saplings, only a weak up-regulation of this gene was seen at late time points after the onset of ozone fumigation. This up-regulation occurred as a result of macroscopic leaf lesion formation (Fig. 4). Genes expressed with lesion development have been classified as late ozone-responsive (Langebartels et al. 2002). Although this classification has a time scale of 48 h, *DHQS* from beech can be considered as late-responsive within the time scale of this study. Interestingly, the abundance of *DHQS* transcripts in control saplings was the second highest after *DAHPS2*. This indicates that shikimate pathway genes, which are highly constitutively expressed (Fig. 9), are not regulated by environmental factors.

For *DHQD/SD*, which encodes a bifunctional enzyme, a significant induction was observed only after increasing the ozone concentration from 150 to 190 nl l^{-1} (Fig. 4). A previous report showed that *DHQD/SD* was induced by ozone fumigation (300 nl l^{-1}) over a period of 30 days (Betz et al. 2008). In the ozone-sensitive tobacco cultivar Bel W3, maximum transcript levels with a twofold induction were observed 10 h after the onset of fumigation, but a slight increase in the controls was also observed (Janzik et al. 2005), and exposure to continuously increasing ozone concentrations (120 nl l^{-1}; 30 days) was found to increase shikimate dehydrogenase activity in poplar leaves (Cabané et al. 2004). The sevenfold increase in the transcript level in beech was similar to the ninefold increase described for tomato cell cultures upon elicitation (Bischoff et al. 2001). This indicates the important function of this enzyme for shikimate pathway intermediates involved in branch pathways on one hand, and for the synthesis of chorismate on the other. Plants can convert 3-dehydroshikimate into protocatechuic and gallic acids or into chlorogenic acid via quinate, which results in reduced chorismate formation (Herrmann 1995; Mustafa and Verpoorte 2005; Ossipov et al. 2003). Therefore an increased DHQD/SD activity can compensate for this loss. Furthermore it has been shown in *Pinus taeda* that SD can accept quinate as a substrate (Ossipov et al. 2000). An induction of DHQD/SD might then increase shikimate pathway intermediates by conversion of quinate to 3-dehydroquinate. The increased accumulation of DHQD/SD protein by Western blot analysis was in agreement with increased transcript levels (Figs. 4, 5). An increased SD activity was also found in a pathogen-resistant tomato line upon ozone exposure for 3 h (Guidi et al. 2005). This parallel increase of transcript and protein levels indicates again, as for *DAHPS3,* a regulation at the transcriptional level.

SK was weakly, but significantly, induced after 2 days of ozone exposure and increased again after 34 days (Fig. 4; Supplementary material Table S4). In contrast *SK* transcripts levels were not affected at all by ozone in tobacco, at least within the experimental time frame of 35 h (Janzik et al. 2005). Again, this may depend on (1) the plant species studied, (2) different experimental conditions or, as seems most likely, (3) the greater sensitivity of qRT-PCR compared to Northern blotting. In tomato cell cultures SK was strongly up-regulated upon elicitation with *Phytophthora megasperma* within 2 h (Görlach et al. 1995), and elicitation of rice calli resulted in an induction of *SK1* and *SK2* but not of the isoform *SK3* (Kasai et al. 2005). Therefore the induction of *SK* upon abiotic/biotic stress contributes also to the formation of an increased level of shikimate pathway intermediates that may contribute to the formation of defense and signaling metabolites.

EPSPS has been analyzed in much more detail than any of the other shikimate pathway genes. The main reason is its susceptibility toward its inhibitor glyphosate, which is widely used as an efficient selective herbicide. In beech saplings the transcript level of *EPSPS* was elevated by a factor of 2.6 within 2 days after the onset of ozone exposure, and it remained significantly elevated until the end of the exposure period (Fig. 6). A slight induction of *EPSPS* has been observed in ozone-treated tobacco as well as in beech saplings analyzed up to 30 days (Janzik et al. 2005; Betz et al. 2008). Strong transcriptional induction has been observed in tomato cell cultures as well as in maize cell cultures upon elicitation, with increased activities of two EPSPS isoforms (Görlach et al. 1995; Forlani 2002).

The final enzyme of the pathway analyzed in this study, *CS*, was weakly induced, but its induction occurred in parallel with the appearance of leaf symptoms (Fig. 6). In tomato cell cultures having two *CS* isoforms, only *CS1* was increased to a high level (Görlach et al. 1995). In tobacco, *CS* seemed to be induced after a short ozone pulse (Janzik et al. 2005). This indicates similar transcriptional reactions of herbaceous and woody plants upon ozone exposure.

We have shown that all shikimate pathway genes of European beech are up-regulated upon ozone treatment. Induction of all shikimate pathway genes have also been reported for other plants (Görlach et al. 1995; Bischoff et al. 1996, 2001; Kasai et al. 2005). Therefore it seems that ozone acts like an abiotic elicitor on shikimate pathway genes, as already described for other genes and biosynthetic pathways (Sandermann et al. 1998).

CM and phenolic metabolites

Chorismate mutase is the branch enzyme toward phenylalanine and tyrosine formation. An induction of *CM* has been reported in *A. thaliana* after wounding and elicitation, and increased enzymatic activity has been described in oat (Mobley et al. 1999; Matsukawa et al. 2002). Although *CM* was significantly induced in beech leaves exposed to ozone (Fig. 6), only a weak increase in soluble and cell wall-bound phenolic metabolites was observed (Fig. 8). Phenolics derived from phenylalanine are a large group of secondary metabolites with an aromatic structure (Heller 1994), and their biosynthesis is therefore extensively regulated. Ozone-induced levels of phenolics have been found in many plants species, including trees (Rosemann et al. 1991; Langebartels et al. 1997; Saleem et al. 2001; Cabané et al. 2004; Kontunen-Soppela et al. 2007). Independent of ozone exposure, the total amount of soluble phenolics decreased during leaf development, and the level of cell wall-bound fraction rose accordingly (Fig. 8). A similar behavior has been described in 60-year-old European beech in a free-air chronic ozone field study (Bahnweg et al. 2005), as well as in mature Scots pine and Norway spruce trees, where a deposition of the soluble fraction into cell walls during leaf ontogeny has been suggested (Heilemann and Strack 1990; Kaffarnik et al. 2006).

In the present study, we observed the appearance of 3,3′,4,4′-tetramethoxybiphenyl in response to ozone exposure. This compound has previously been detected in pathogen-affected and ozone-treated leaves of beech (Zielke and Sonnenbichler 1990), and has been termed a stress-metabolite in beech because of its accumulation in *Apiognomomia errabunda*-infected leaves (Bahnweg et al. 2005). Interestingly, the reduction of the chlorogenic acid level in ozone-treated leaves extended beyond the respective ontogenetic level in leaves of control saplings in our study. This effect has also been reported from ozone-treated tobacco (Janzik et al. 2005) and can be explained by oxidative degradation or deposition of chlorogenic acid in the presence of ozone. Although these ozone-induced changes in the amounts of phenolics were not significant, the weak accumulation of soluble and cell wall-bound phenolics is in agreement with an increased transcription of *CM*.

SA and gentisic acid GA

Salicylic acid is an important signal molecule in plant defense (Shah 2003; Métraux and Durner 2004). It is also involved in the plant's response to ozone (Sharma et al. 1996; Rao et al. 2002; Kangasjärvi et al. 2005). It is well known that SA can be formed from phenylalanine via cinnamate and benzoate, or more directly from chorismate via isochorismate (Wildermuth et al. 2001; Métraux 2002; Shah 2003; Mustafa and Verpoorte 2005). Although levels of free SA are rather constant in beech leaves regardless of age or treatments, conjugated SA increased significantly upon ozone exposure in our study (Fig. 7). In a free-air chronic ozone exposure experiment with adult European beech trees in a natural stand, increased levels of conjugated SA were found in sun leaves (Nunn et al. 2005a). It has been demonstrated that ozone-induced accumulation of PR proteins is SA-dependent (Sharma et al. 1996). This is in agreement with increased levels of conjugated SA as well as increased *PR* transcript levels in beech leaves in several studies (Betz 2005; Olbrich et al. 2005).

GA is synthesized via hydroxylation of SA (Mustafa and Verpoorte 2005). In addition to conjugated SA we also detected increased levels of conjugated GA (Fig. 7), as has earlier been described for beech (Nunn et al. 2005a). Pathogen infection of tomato also results in an accumulation of conjugated GA together with PR proteins (Bélles et al. 1999). Since both conjugated SA and GA are products of shikimate metabolism, their accumulation is consistent with increased transcription of shikimate pathway genes.

Conclusions

Our study clearly demonstrates an ozone-dependent reprogramming of the transcription of all shikimate pathway genes, which have effects on secondary metabolism. Different isoforms of these genes in beech seem to regulate the pathway differently, as has been similarly demonstrated for herbaceous plants. The expression of two of the pathway genes was concurrently analyzed at the transcript and protein levels, and the two types of experiment are in agreement with each other. The increased transcriptional level of all shikimate pathway genes may explain the formation of conjugated SA and GA, both well-known for their role in mediating stress responses in plants. Whether their formation occurs directly via chorismate or phenylalanine is not yet clear.

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