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Temporal regulation of terpene synthase gene expression in *Eucalyptus globulus* leaves upon ozone and wounding stresses: relationships with stomatal ozone uptake and emission responses

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Abstract

Ozone and wounding are key abiotic factors but, their interactive effects on temporal changes in terpene synthase gene expression and emission responses are poorly understood. Here, we applied combined acute ozone and wounding stresses to the constitutive isoprenoid-emitter *Eucalyptus globulus* and studied how isoprene, 1,8-cineole, and isoleudene synthase genes were regulated, and how the gene expression was associated with temporal changes in photosynthetic characteristics, product emission rates, and stomatal ozone uptake through recovery phase. Photosynthetic characteristics and emission rate of isoprene, 1,8-cineole, and isoleudene were synergistically altered, while three TPS gene expressions were antagonistically altered by combined stress applications. A time-delay analysis indicated that the best correspondences between gene expression and product emission rates were observed for 0 h time-shift for wounding and 0–2 h time-shifts for separate ozone, and combined ozone and wounding treatments. The best correspondence between ozone uptake and gene expression was observed for 0–4 h time-shifts for

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Author's contribution

AK - Conception and design, execution of experiment, analysis and interpretation of the data, drafting of the article, and critical revision of the article for important intellectual content.

LP - Execution of experiment and drafting of the article.

RB - Execution of experiment and interpretation of the data

CK - Execution of experiment.

ÜN - Drafting of the article and interpretation of data.

Conflicts of interest

None.

separate ozone and combined ozone and wounding treatments. Overall, this study demonstrated that expression profiles of isoprene, the monoterpene 1,8-cineole, and the sesquiterpene isoprene synthase genes differentially influenced their corresponding product emissions for separate and combined ozone and wounding treatments through recovery.

Keywords

Acute ozone stress; antagonistic effect; 1,8-cineole synthase; isoprene synthase; recovery kinetics; stomatal ozone uptake; synergistic effect; terpene synthase gene expression; time-delay analysis

1 Introduction

Ozone is a key air pollutant and a plant stress elicitor. It is mainly formed in the troposphere when nitrogen oxides (NO_x) react with reactive volatile organic compounds (VOCs) in the presence of sunlight (Ryerson et al., 2003). Currently, the tropospheric ozone concentration in the most parts of the world (35–50 ppb in the Northern Hemisphere) can still cause biochemical alterations and physiological damage in plants (Sicard et al., 2017), especially in fast-growing herbaceous species and deciduous trees (Huttunen and Manninen, 2013; Sicard et al., 2017). It was predicted that the current surface level **ozone** may increase by 20–25% between 2015 and 2050, and by 40–60% in 2100, primarily due to increasing rate of industrialization and burning of fossil-fuels (Huttunen and Manninen, 2013; Vingarzan, 2004).

In natural environments, plants often face multiple stress factors affecting plants simultaneously or sequentially (Copolovici et al., 2014; Niinemets, 2010c). In addition, the occurrence of multiple stress factors with varying strength and duration significantly influences the physiological and biochemical processes of plants through additive, synergistic, and antagonistic interactions (Niinemets, 2010c). Elevated ozone exceeding a certain threshold exerts oxidative stress in plants, resulting in reduced plant growth primarily through curbing photosynthetic processes (Hartikainen et al., 2012; Li et al., 2017).

Stomatal uptake is the primary channel through which atmospheric ozone enters leaf intercellular spaces and causes oxidative damage in plant cells (Beauchamp et al., 2005; Heath, 2008). Besides the changes in key metabolic activities such as photosynthesis and plant growth, ozone exposure leads to substantial alterations in the rate of emission and composition of plant volatile blend both during initial stress applications and through recovery (Beauchamp et al., 2005). Wounding is a mechanical stress factor that primarily results from the impacts of herbivore feeding, wind, moving objects, and precipitation (Benikhlef et al., 2013; Portillo-Estrada et al., 2015). Similar to elevated ozone, wounding also affects primary and secondary metabolic processes of plants due to curbing photosynthetic traits and altering volatile emission responses (Brilli et al., 2011; Kanagendran et al., 2018; Loreto and Sharkey, 1993; Portillo-Estrada et al., 2015).

Under non-stressed conditions, plants emit volatiles constitutively; however, stress episodes exponentially elicit volatile emission responses, particularly LOX volatiles (lipoxygenase

pathway volatiles, also called green leaf volatiles, GLV) and volatile isoprenoids (Copolovici et al., 2014; Pazouki et al., 2016). Furthermore, elevated ozone (Beauchamp et al., 2005; Li et al., 2017; Llusia et al., 2002; Loreto et al., 2001; Loreto et al., 2004; Peñuelas et al., 1999; Velikova et al., 2005) and wounding (Copolovici et al., 2017; Portillo-Estrada et al., 2015) substantially influence the activity of lipoxygenase (LOX), 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate (MEP/DOXP), and mevalonate (MVA) pathways and in turn, they alter LOX and volatile isoprenoid emission responses.

Eucalyptus spp. is native to Australian forests. It is a valuable hardwood for pulp industry, sawmills, and biofuels. Due to their high growth rates, excellent form, and outstanding hardwood properties, they belong to economically most important plant species in the world (Külheim et al., 2015). In addition, *Eucalyptus* species are of great ecological value as they are strong isoprenoid emitters even under non-stressed conditions (Funk et al., 2006; Guenther et al., 1991; Loreto et al., 2000; Winters et al., 2009). To the best of our knowledge, there is a scarcity of information of time-dependent terpene synthase (TPS) gene-level regulation of volatile emission responses in eucalypt species upon different stresses.

The terpene synthase gene family of eucalypt falls into three classes and seven sub-families: Class I consists of TPS-c (copalyl diphosphate and ent-kaurene), TPS-e/f (ent-kaurene and other diterpenes as well as some mono- and sesquiterpenes) and TPS-h (*Selaginella* specific); class II consists of TPS-d (gymnosperm specific) and class III of TPS-a (sesquiterpenes), TPS-b (cyclic monoterpenes and hemiterpenes) and TPS-g (acyclic monoterpenes) (Chen et al., 2011; Külheim et al., 2015). In Tasmanian blue gum (*E. globulus* Labill.), there are 106 putatively functional TPS genes responsible for the biosynthesis of volatile and non-volatile isoprenoids (Külheim et al., 2015). Expression of TPS genes was observed in different parts of *E. grandis* and particularly, genes of TPS-a, TPS-b1 and TPS-b2 subfamilies biosynthesizing mono-, sesqui- and hemiterpenes were shown to be significantly expressed in leaves (Külheim et al., 2015).

In this study, we used *E. globulus* as a reference plant species widely investigated in isoprenoid emission studies (Guenther et al., 1991; Loreto et al., 2000). Yet, the relationships between temporal regulation of terpene synthase gene expression and volatile emission responses upon different stresses have not been studied in this species. In particular, there is no information about temporal regulation of terpene synthase genes in response to ozone and wounding treatments and their relationship with stomatal ozone uptake and the rate of terpene emission. In *E. globulus*, three genes are of special importance for foliage terpenoid emission: isoprene synthase (TPS-b2 terpene synthase subfamily), 1,8-cineole synthase (TPS-b1), and isodene synthase (TPS-a). We demonstrated that there was a great variation of emission responses of isoprene, 1,8-cineole, and isodene through recovery upon separate and combined ozone and wounding treatments (Kanagendran et al., 2018).

The key objectives of the current study were to (1) study the potential impact of photosynthetic-derived substrate level controls on temporal emission rates of isoprene, 1,8-cineole, and isodene, (2) relate the expression profiles of isoprene, 1,8-cineole, and

isolekene synthase genes with product emissions through recovery, (3) investigate the time-delay correlation between isoprene, 1,8-cineole, and isolekene synthase gene expression levels and the emission, and (4) analyze the time-delay correlation between ozone uptake rate and expression profiles of isoprene, 1,8-cineole and isolekene synthase genes to assess the lag-time between ozone exposure and corresponding changes in gene expression.

We hypothesized that (1) temporal changes in photosynthetic traits will influence the emission rates of isoprene, 1,8-cineole, and isolekene through changes in substrate availability upon separate and combined ozone and wounding treatments, (2) changes in relative expression levels of terpene synthase genes will be greater for combined ozone and wounding treatments, followed by separate ozone and wounding treatments through recovery (3) combined applications of ozone and wounding treatments will synergistically alter the expression and emission of isoprene, 1,8-cineole, and isolekene and that the synergistic effect will scale with the severity of stress applications, and (4) the time-delay among ozone uptake, gene expression and product emission varies with the different stress applications and terpene synthase gene clades.

2 Materials and Methods

2.1 Plant material and growth system

Eucalyptus globulus seeds (seed source: OMC seeds Ltd., Lithuania) were sown in 5 L pots filled with 1:1 mixture of quartz sand and commercial potting soil (Kekkilä group, Finland). The soil water pH through the experimental period was 6.2. The plants were fertilized with macronutrients N (100 mg L⁻¹), P (30 mg L⁻¹), and K (200 mg L⁻¹) and all necessary micronutrients. The seedlings were grown for three weeks in a growth chamber (Percival AR-95 HIL, CLF Plant Climatics GmbH, Wertingen, Germany) under controlled environmental conditions as follows: light intensity at leaf surface of 400-500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with 12 h photoperiod, chamber temperature (day/night) of 28/25 °C, relative humidity of 60-70 %, and ambient CO₂ concentration of 380-400 $\mu\text{mol mol}^{-1}$. Three-week-old seedlings were transplanted into 10 L pots containing the same potting mixture and kept in a plant growth room under similar environmental conditions.

Each plant was watered every two days and fertilized once a week with 80 ml liquid fertilizer (Baltic Agro, Lithuania) (ca. 0.4 % solution) (NPK ratio: 5:5:6, and micronutrients: B (0.01%), Cu (0.03%), Fe (0.06%), Mn (0.028%), and Zn (0.007%)) for optimal plant growth. In all experimental treatments, one-year-old plants with ca. 1 m tall plants of a similar biomass were selected and only non-senescent leaves were used for the experimental treatments.

2.2 Experimental set-up, gas-exchange measurements, and volatile collection and GC-MS analysis

We used a custom-made gas exchange system for ozone fumigation, gas-exchange measurements, and volatile collection. The system had a double-layered cylindrical glass chamber (1.2 L) that was illuminated by four 50 W halogen lamps, intensity of which was controlled by a regulatory unit. The chamber temperature was controlled by circulating

distilled water between the double layers of the glass chamber using a circulating water bath. Air temperature inside the chamber was measured by a thermistor (NTC thermistor, model ACC-001, RTI Electronics, Inc., St. Anaheim, CA, USA), and leaf temperature by a thermocouple attached to the lower leaf surface. Ambient air passing through a charcoal filter and custom-made ozone trap (passing less than 2 ppb ozone) was used. A fan (Sunon Group, Beijing, China) inside the leaf chamber was used to obtain high air turbulence at a moderately low wind speed. Internal surface of all chamber connections and tubing was coated with stainless steel and Teflon® to minimize the memory effects due to adsorption on and release of volatiles from system components (Niinemets et al., 2011).

Gas-exchange measurements and volatile sampling were started after stabilization of gas flows and leaf gas-exchange rates, ca. 10-15 min after leaf enclosure in the chamber. The first set of measurements was completed prior to experimental treatments (time 0). The measurements were repeated at 0.5 and 3, 10, 25, 50, and 75 h after stress treatments. A separate analysis indicated that foliage gas-exchange and volatile emission rates of control samples were consistent (variation less than 5%) during the entire experimental period. Ozone was produced at a stable rate by Certizon C100 ozonizer (Erwin Sander Elektroapparatenbau GmbH, Germany) when required. The ozone concentration at the chamber in- and outlet was measured using a UV photometric ozone detector (Model-49i, Thermo Fisher Scientific, Franklin MA, USA). The chamber inlet and outlets were switched between the reference and measurement modes for gas exchange measurements and volatile sampling. Changes in CO₂ and H₂O concentrations at chamber in- and outlets were estimated by an infrared dual-channel gas analyzer operated in reference and measurement modes (CIRAS II, PP-systems, Amesbury, MA, USA). Net assimilation rate and stomatal conductance to water vapor per unit leaf area were calculated as described by von Caemmerer and Farquhar (1981).

The volatiles were collected into stainless steel cartridges filled with three different types of adsorbent materials such as carbotrap C 20-40 mesh, carbopack B 40-60 mesh, and carbotrap X 20-40 mesh (Kännaste et al., 2014). The volatiles from glass chamber were collected through chamber outlet. In fact, 4 L of air from glass chamber were passed through stainless steel cartridges during 20 minutes of volatile sampling at a constant suction rate of 200 ml / min, maintained by a portable suction pump 210-1003 MTX (SKC Inc., Houston, TX, USA). A two-step desorption method were employed to analyze the stainless steel cartridges in GC-MS. In the first step, He purge flow was set to 40 ml / min, primary desorption temperature to 250 °C, and primary desorption time to 6 min. In the second step, a trap temperature during primary desorption was set to -20 °C, and second stage trap desorption temperature to 280 °C withhold time of 6 min (Kännaste et al., 2014).

GC oven program used for separation of the volatile compounds was as follows: 40 °C for 1 min, 9 °C / min to 120 °C, 2 °C / min to 190 °C, 20 °C / min to 250 °C, and 250 °C for 5 min. The mass spectrometer of GC-MS instrument was operated in electron-impact mode (EI) at 70 eV, in the scan range of m/z 30-400. The transfer line temperature was set at 240 °C and ion-source temperature at 150 °C. Compounds were identified by the NIST library of mass spectra and based on retention time identity with the authentic standards (GC purity) (Kännaste et al., 2014). The emission rates of volatiles were estimated according to

Niinemets et al. (2011). The background (blank) volatile concentrations were subtracted from the emission samples with the leaves to estimate the net foliage emission rate of volatiles per unit leaf area. Further details about the same experimental set-up for ozone fumigation, gas exchange, volatile samplings, and GC-MS analysis is provided by Kanagendran et al. (2018) and Kännaste et al. (2014).

2.3 Stress application protocol

As a strong isoprenoid emitter, *E. globulus* show a significant resistant to acute ozone exposures, since a considerable quantity of ozone is scavenged by higher concentration of volatile isoprenoids in ambient air, leaf intercellular air spaces, and leaf boundary layer and by water-soluble antioxidants such as putrescine and apoplastic ascorbate within the aqueous phase in cell walls (Bouvier-Brown et al., 2009; Fares et al., 2010; Goldstein et al., 2004; Kurpius and Goldstein, 2003). Furthermore, a previous report demonstrated that a continuous exposure of 0.8 ppm ozone for 6 h did not even cause physiological perturbations in *E. globulus* leaves and thus, *E. globulus* are categorized as “extremely resistant” plant species to acute ozone exposures (O'connor et al., 1975). In addition, a preliminary study indicated that exposure of *E. globulus* leaves to 0.3-2 ppm ozone did not cause a considerable variation in photosynthetic characteristics and LOX emission rates (Kanagendran et al., 2018). Therefore, we used higher acute ozone exposures in this study. In fact, we observed that ozone stress influences plants on ozone-dose (ozone-sum) dependent manner that is dependent on both the concentration and the duration of ozone exposures (ozone concentration above a threshold limit X exposure time) (Beauchamp et al., 2005). Nevertheless, chronic ozone stress impacts plants quite differently due to secondary acclimation responses (Beauchamp et al., 2005; Calfapietra et al., 2007), we consider the higher acute ozone exposures as a representative for a quantitative characterization of plant responses to different ozone stress levels (Li et al., 2017).

In this study, four sets of experiments were carried out: control (no treatment), separate wounding (7 cm length of cut), separate ozone exposures (three different severity of ozone exposures of 4, 5 and 6 ppm), and combined ozone and wounding treatments, where ozone exposures (the same three different ozone exposures) were rapidly followed by wounding treatment. A randomly selected branch with 6 fully mature leaves was used for each experiment, and the constant baseline conditions maintained in the glass chamber were: light intensity at the leaf surface of 700-750 $\mu\text{mol m}^{-2} \text{s}^{-1}$, chamber temperature of 25 °C (leaf temperature of 25-27 °C), ambient CO₂ concentration of 380-400 $\mu\text{mol mol}^{-1}$, and relative air humidity of 60-70%. For wounding treatments, four holes were rapidly created (within 6 s) in each leaf lamina by a paper punch. The area of each disc detached was ca. 25 mm² and thus, the total perimeter length (wound edge of four holes) created was 7 cm (see Portillo-Estrada et al. (2015) for detailed methodology of wounding). For separate ozone treatments, a branch consisting of 6 leaves was exposed to either 4, 5, or 6 ppm ozone for 3 hours. In the case of combined ozone and wounding treatments, the branch was removed from glass chamber after ozone exposures, and four punch holes were instantly created (within 6 s) in each leaf as for the treatment with wounding alone.

2.4 Measurement of chlorophyll fluorescence and leaf area

Measurements of maximum dark-adapted (10 min darkening) quantum yield of photosystem II (PSII, F_v/F_m) of treated and untreated leaf were conducted with a PAM fluorometer (Walz IMAG-MIN/B, Walz GmbH, Effeltrich, Germany). The harvested leaves were scanned at 300 dpi to measure leaf area using a custom made software tool.

2.5 Estimation of ozone uptake

Average stomatal conductance for ozone for the entire 3 h ozone exposure (g_{s,O_3}) was estimated as the average stomatal conductance for water vapor ($g_{s,w}$) divided by the ratio of the binary diffusion coefficients for ozone and water vapor (2.03; see Li et al. (2017) for details). Average stomatal ozone uptake rate ($\Phi_{O_3,s}$) was calculated as the product of average chamber ozone concentration for the entire 3 h exposure and g_{s,O_3} for ozone exposures. In this calculations, we assumed that ozone uptake through the cuticular layer was negligible (Kerstiens and Lenzian, 1989) and that the intercellular ozone concentration was zero (Laisk et al., 1989). In practice, the second assumption may produce somewhat overestimated stomatal ozone uptake rates, since ozone concentration in the leaf intercellular air space are marginally higher than zero (Moldau and Bichele, 2002).

2.6 Collection of tissue samples, RNA extraction, and cDNA synthesis

Upon completion of leaf area measurements, the leaf tissues collected at 0.5, 3, 10, 25, 50 and 75 h after completion of experimental treatments were immediately transferred to liquid nitrogen and kept at -80°C until RNA extraction was carried out. Leaf tissues were homogenized by mortar and pestle in liquid nitrogen, followed by RNA extraction using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. In addition, RNA concentration of all RNA samples was estimated using Biophotometer Plus (Eppendorf, Germany) and when needed, it was adjusted with nuclease-free water. cDNA was synthesized from one microgram of RNA using Maxima First Strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, USA).

2.7 qPCR primer design and reaction conditions

Two reference genes, cyclin-dependent kinase E-1 (*Eucon04*, Genbank accession number EEF43392.1) and transcription elongation factor s-II (*Eucon08*, Genbank accession number EEF33688.1) were used as reference genes to normalize gene expression following de Oliveira et al. (2012). Both genes demonstrated highly stable expression rates over all treatment and control samples. We selected three representative TPS genes, expression of which has been demonstrated to occur *in vivo* in eucalypt leaves (Külheim et al., 2015).

The primers for isoprene synthase (XM_010037321.2), 1,8-cineole synthase (DD464633.1), and isodene synthase genes (Külheim et al., 2015) were designed using Primer3Plus (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>). The length of all primers was between 20-26 bp and the melting temperature was between 50 and 57 °C. Details of oligonucleotide primers are shown in Table 1.

To examine the eucalypt TPS gene expression in response to ozone and wounding treatments, qPCR was carried out with ViiA™ 7 qPCR System (Applied Biosystems,

Courtaboeuf, France) with a final volume of 33 μ L containing 16.2 μ L (2x) SYBR green PCR master mix (Thermo Fisher Scientific, Waltham, USA), 500 nM of each primer, and 1.5 μ L of 5-fold-diluted cDNA. The qPCR reactions were run in an ABI Sequence Detection System (Applied Biosystems) using the following program: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All qPCR reactions were run using three technical replicates and three biological replicates. A dissociation curve was generated to examine any potential nonspecific amplification and the dissociation program was created as follows: 95 °C for 15 s, 60 °C for 15 s followed by 20 min of slow temperature ramp up from 60 °C to 95 °C. The assay efficiency of three TPS genes was between 85-110%. Relative gene expression levels were quantified based on 2^{-CT} method defined by Livak and Schmittgen (2001).

2.8 Statistical analysis and data handling

In this study, there were four replicates for controls and three replicates for all other treatments with different plants. The individual and interactive effects of ozone and wounding treatments, and recovery time on the relative expression levels of isoprene, 1,8-cineole, and isoleudene synthase genes were statistically tested by generalized linear model (GLM) based on maximum likelihood model fitting. Data for GLM were tested for normality of distribution and homogeneity of variance and when required, the data were log-transformed.

Linear correlation analysis (Pearson) was used to assess for the relationships between ozone uptake rate and relative gene expression levels at different times (0.5-25h) after stress applications. As there is a certain time needed for elicitation of gene expression to protein synthesis and concomitant emission of corresponding volatiles, a time-delay (1-6 h) analysis was employed to examine the relationships between relative gene expression levels and product emission rates of isoprene, 1,8-cineole, and isoleudene. The lag-time was varied between 1-6 h for different treatments and lag-times yielding the highest correlation between emission and gene expression were selected. Similarly, a time-delay (1-6 h) analysis of the relationship between stomatal ozone uptake rates and the relative gene expression levels of isoprene synthase, 1,8-cineole synthase, and isoleudene synthase was also conducted. In addition, a functional characterization of isoleudene synthase gene indicated that it is a multi-product enzyme, primarily producing isoleudene (21.14%), β -cadinene (14.09%), unidentified sesquiterpenes (16.77%), (*Z*)-muurola-3,5-diene (9.06%), β -caryophyllene (8.05%), α -cubebene (6.38%) as well as smaller amounts of α -copaene (2.35%), α -gurjunene (1.68%), α -humulene (3.02%), allo-aromadendrene (1.68%), (*E*)-cadin-1(6),4-diene (3.36%), α -amorphene (4.7%), α -muurolene (2.68%) and germacrene D (5.03%) (Külheim et al. unpublished). However, in our study, emissions of isoleudene, β -cadinene and α -copaene were strongly correlated ($r > 0.60$ for β -cadinene and α -copaene) with isoleudene synthase gene expression in all treatments. Therefore, the expression of isoleudene synthase gene was correlated with the sum of emissions of isoleudene, β -cadinene, and α -copaene in our study.

The correlation analyses were conducted with SigmaPlot 12.5 (Systat Software Inc, San Jose, CA, USA). All other statistical analyses were conducted with SPSS 24 (IBM SPSS, Chicago, IL, WA). All statistical effects were considered significant at $P < 0.05$.

3 Results

3.1 Changes in photosynthetic characteristics versus emission rates of isoprene, 1,8-cineole, and isoprene in response to ozone and wounding treatments

Separate ozone and combined ozone and wounding treatments caused a substantial decline in maximum dark-adapted photosystem II (PSII) quantum yield estimated by chlorophyll fluorescence (F_v/F_m), net assimilation rate and stomatal conductance to water vapor throughout the recovery phase (Fig. S1). Generally, changes in the emission rates of isoprene, 1,8-cineole, and isoprene were inversely related with the changes in F_v/F_m (Fig. S1A and S2). In addition, further details about the changes in gas exchange characteristics and the emission rates of isoprene, 1,8-cineole, and isoprene for the same experimental treatments can be found in our observational study by Kanagendran et al. (2018).

3.2 Expression profiles of terpene synthase genes and their relationship with terpenoid emissions in *E. globulus* leaves upon ozone and wounding treatments

Separate ozone, and combined ozone and wounding treatments suppressed isoprene synthase gene expression levels compared to controls throughout the recovery phase (Fig. 1A). In addition, isoprene synthase gene expression was downregulated till 3 h after separate ozone, and combined ozone and wounding treatments and then it was upregulated till 75 h of recovery. Compared with the control leaves, expression levels of isoprene synthase gene remained lower throughout the recovery phase for 6 ppm ozone, combined 4 ppm ozone and wounding, and combined 6 ppm ozone and wounding treatments. Furthermore, the relative expression level of isoprene synthase gene initially peaked at 10 h, followed by a second peak at 25 h for combined 5 ppm ozone and wounding treatment. Wounding treatment alone resulted in upregulated isoprene synthase gene expression between 0.5-50 h of recovery with the highest expression observed at 25 h of recovery (Fig. 2A, Table 2). There was almost a complete recovery of isoprene synthase gene expression at 75 h of recovery for wounding, 4 ppm ozone, 5 ppm ozone, and combined 5 ppm ozone and wounding treatments. Collectively, these modifications implied that separate and combined ozone and wounding treatments, and recovery time statistically impacted ($P < 0.001$) isoprene synthase gene expression (Table 2).

All stress applications resulted in downregulated 1,8-cineole synthase gene expression levels throughout the recovery phase ($P < 0.001$ for ozone, wounding, combined ozone and wounding treatments, and recovery time), but a lower degree of downregulation was observed in response to wounding treatment alone and a higher degree of downregulation was observed for 6 ppm ozone, and combined 6 ppm ozone and wounding treatments at all recovery times (Fig. 1B and Table 2). Similar to the expression response of isoprene synthase gene, the lowest levels of 1,8-cineole synthase gene expression were observed till 3 h of recovery and then the expression slightly increased till 75 of recovery. There was no

recovery of 1,8-cineole synthase gene expression throughout the recovery phase (Fig. 1B, Table 2).

In contrast to the expression of isoprene and 1,8-cineole synthase genes, wounding progressively enhanced isoprene synthase gene expression levels throughout the recovery phase ($P < 0.001$ for wounding), but the gene expression levels in response to separate ozone and combined ozone and wounding treatments greatly varied through the recovery phase ($P > 0.05$ for separate ozone, combined ozone and wounding treatments, and recovery time) at all recovery times (Fig. 1C and Table 2). The relative expression level of isoprene synthase gene was downregulated for 4 ppm ozone, combined 4 ppm ozone and wounding, and combined 5 ppm ozone and wounding treatments, but it was upregulated for separate 5 ppm and 6 ppm ozone treatments compared to controls throughout the recovery phase. However, the application of 6 ppm ozone treatment led to upregulated isoprene synthase gene expression levels throughout the recovery phase. Expression of isoprene synthase gene did not reach the pre-stress level, except for combined 6 ppm ozone and wounding treatments at 75 h of recovery phase (Fig. 1C).

A regression analysis indicated that the expression responses of isoprene and 1,8-cineole synthase genes were correlated throughout the recovery phase upon all treatments ($P < 0.05$, Fig. S3). In contrast, the expression profiles of isoprene synthase were not correlated with the expression profiles of both isoprene and 1,8-cineole synthase genes in any of the stress applications in any of the recovery times ($P > 0.05$, Fig. S4 and S5).

Negative correlations among isoprene synthase gene expression and isoprene emission were observed for all separate ozone and wounding treatments, and for combined 6 ppm ozone and wounding treatments, and positive correlations were observed for combined 4 ppm ozone and wounding, and combined 5 ppm ozone and wounding treatments (Fig. 2). The correlations between 1,8-cineole synthase gene expression and 1,8-cineole emission were negative for all other stress applications (Fig. 3A, Fig. 3B, and Fig. 3C), except for wounding, where it was positive (Fig. 3D). Furthermore, the correlations between isoprene synthase gene expression and total emission rate of primary products of isoprene synthase gene (isoprene, α -cadinene, and α -copaene) were negative for 4 ppm ozone, and combined 4 ppm ozone and wounding treatments and positive for all other stress applications (Fig. 4).

The time-lag analyses with 1-6 h time-shift indicated that usually, the best correspondence between TPS gene expression and emission was observed for 0 h time-shift for wounding treatments (Fig. 2D, Fig. 3D, and Fig. 4D) and 0-2 h time-shift for separate ozone, and combined ozone and wounding treatments (Fig. 2-4 for 0 h time-shift and Table 3). In particular, the introduction of lag-time between relative gene expression and emission rates of isoprene, 1,8-cineole, and isoprene upon 6 ppm ozone treatment resulted in the strongest correlations for time-shifts of 1-6 h (Table 3). Generally, most of the cases, these correlations were stronger for severe stress applications, particularly for combined ozone and wounding treatments followed by higher doses of separate ozone treatments for all compounds (Fig. 2-4 and Table 3).

3.3 Relationships between stomatal ozone uptake rates and terpene synthase gene expression levels across ozone and wounding treatments

In most of the cases, there were negative correlations between stomatal ozone uptake rates and expression profiles of isoprene, 1,8-cineole, and isoprene synthase genes upon separate ozone, and combined ozone and wounding treatments at 0.5-25 h of recovery time (Fig. 5-7). However, the correlations between stomatal ozone uptake rates and isoprene synthase gene expression levels were positive for 5 ppm and 6 ppm ozone treatments. In all cases, the correlations between stomatal ozone uptake rates and isoprene synthase gene expression were significant (Fig. 7).

Based on the time-delay analysis, the correlations between stomatal ozone uptake rates and gene expression levels of isoprene, 1,8-cineole, and isoprene synthase genes were enhanced by introducing shifts of 1-6 h in ozone uptake rates (Table 4). However, in most of the cases, the strongest correlations were observed for 0-4 h of time-shift, particularly for 6 ppm ozone, and for combined 4 ppm ozone and wounding and combined 5 ppm ozone and wounding treatments (Fig. 5-7 and Table 4). Independent of the time-lag, the strongest correlations between ozone uptake rates and terpene synthase gene expression were observed in response to 6 ppm ozone treatment (Fig. 5-7 and Table 4).

4 Discussion

4.1 Impact of photosynthetic characteristics on the emission of volatile isoprenoids upon ozone and wounding treatments

Isoprene and monoterpenes are biosynthesized via the MEP/DOXP pathway in plastids and sesquiterpenes via the MVA pathway in cytosol (Pazouki and Niinemets, 2016). Therefore, the activity of MEP/DOXP pathway in mesophyll is primarily dependent on the constant supply of NADPH and ATP generated by photosynthetic electron transport (Niinemets et al., 2002; Rasulov et al., 2016). Therefore, isoprene and monoterpene emission rates are often correlated with net assimilation rate, but there is no similar relationship required for sesquiterpene emission (Niinemets et al., 2002; Staudt and Lhoutellier, 2011). In fact, the emission rate of isoprene from control leaves was comparatively low ($1.4 \pm 0.8 \text{ nmol m}^{-2} \text{ s}^{-1}$) (Fig. S2A), primarily associated with lower leaf dry mass per unit area (LMA) of young *E. globulus* plants used in our study (Harley et al., 1997). In our study, compared to controls, emissions of isoprene and 1,8-cineole were remained higher, while net assimilation rate was remained lower throughout the recovery phase in response to ozone and wounding treatments (Fig. S1B and Fig. S2A and S2B) (Kanagendran et al., 2018). Therefore, substrate-level control, for example DMADP for isoprene and GDP for 1,8-cineole, was not substantial for the biosynthesis of isoprene and 1,8-cineole. In addition, the reduction of isoprene emission upon wounding was also associated with the reduction of net assimilation rate and thus, substrate-level control could be plausible (Fig. S1B and Fig. S2A) (Kanagendran et al., 2018). However, in our study, we cannot rule out the enzymatic control acting along with substrate level control in all cases. As sesquiterpenes, isoprene, α -cadinene, and α -copaene emissions are not dependent on the supply of photosynthetic intermediates. In addition, the emission rate of hydrophilic oxygenated monoterpene, 1,8-cineole, is partly regulated by stomatal conductance, while the emission of non-oxygenated

volatile isoprenoids such as isoprene, isoleudene, γ -cadinene, and α -copaene is independent of stomatal conductance (Fall and Monson, 1992; Niinemets et al., 2002). Therefore, the reduced stomatal conductance could have reduced time-dependent emission of 1,8-cineole to some extent (Fig. S1C and Fig. S2B) (Kanagendran et al., 2018). Furthermore, in our study, moderate stomatal closure could likely increase isoprene emission to a certain extent as a result of positive effect of reduced intercellular CO₂ concentration on isoprene biosynthesis (Rasulov et al., 2016) (Fig. S1C and Fig. S2A).

4.2 Expression profiles of studied terpene synthase genes in response to ozone and wounding treatments

Analysis of transcript abundance of isoprene (TPS-b2), 1,8-cineole (TPS-b1), and isoleudene synthase (TPS-a) genes demonstrated that the expression profiles of terpene synthase genes were differentially regulated through the recovery phase (Fig. 1A-C). The relative expression levels of isoprene, 1,8-cineole, and isoleudene synthase genes were significantly altered by separate and combined ozone and wounding treatments throughout the recovery phase (Fig. 1A-C). Therefore, it is suggested that a combined effect of ozone and wounding treatments on terpene synthase gene expression was different from that of separate ozone and wounding treatments.

Expression profile of isoleudene synthase gene demonstrated the highest gene-responsiveness, followed by 1,8-cineole and isoprene synthase genes for all stress application (Fig. 1A-C). In addition, the responsiveness of all three TPS genes was highest for wounding, followed by separate ozone, and combined ozone and wounding treatments throughout the recovery phase. Taken together, these findings suggest that there are different stress-stimulus responsive regions in three TPS synthase genes studied and thus, they were differentially responded upon different abiotic stressors. Several studies reported the differential regulation of terpene synthase genes upon abiotic and biotic stresses. In accordance with our findings, Steele et al. (1998) reported that mechanical wounding upregulated terpene synthase gene expression. Furthermore, application of acute heat stress on tomato (*Solanum lycopersicum*) leaves downregulated β -phellendrene and β -caryophyllene synthase genes (Pazouki et al., 2016) and in another study, drought stress downregulated terpene synthase gene expression in aleppo pine (*Pinus halepensis*) (Fox et al., 2018). However, exogenous application of methyl jasmonate to norway spruce (*Picea abies*) trees led to increased transcript accumulation of mono- and diterpenoid synthases in stem tissues (Fäldt et al., 2003). In addition, UV-B irradiation differentially alters the transcript abundance of terpene synthase genes in melting flesh peach (*Prunus persica*) fruits and application of jasmonic acid upregulated sesquiterpene synthase gene expression in peach fruits (Liu et al., 2017). Biotic stresses were reported to upregulate terpene synthase gene expression profiles; for example, white pine weevils (*Pissodes strobi*) attack in Sitka spruce (*Picea sitchensis*) (Miller et al., 2005) and feeding of herbivore Gypsy moth (*Lymantria dispar*) in the leaves of western balsam poplar (*Populus trichocarpa*), eventually leading to significant quantity of volatile terpenoid emissions (Irmisch et al., 2014).

As isoprene and 1,8-cineole synthase enzymes are plastidic enzymes, their gene expression profiles are significantly correlated throughout the recovery phase (Fig. S3), implying that

the same transcription factors could have regulated their expression profiles. However, because isoprene synthase is a cytosolic enzyme, there were weak correlations observed among relative expression levels of isoprene synthase and 1,8-cineole synthase genes with isoprene synthase genes (Fig. S4 and Fig. S5). In contrast to our findings, there were strong positive correlations observed between plastidic β -phellandrene synthase and cytosolic β -caryophyllene/ α -humulene synthase genes and product emission rates upon heat-shock treatments in tomato leaves (Pazouki et al., 2016). These results collectively imply the existence of various transcriptional factors differentially regulating TPS gene expression for different abiotic stressors. Further work is needed to characterize the pertinent transcription factors and their regulatory effects on terpene synthase gene expression for acute ozone and wounding stresses.

4.3 Expression profiles of terpene synthase genes versus terpene emission rates upon ozone and wounding treatments

As isoprene is *de novo* biosynthesized (Rasulov et al., 2014; Rasulov et al., 2009; Vickers et al., 2010), the rate of isoprene emission is generally regulated by three factors: substrate availability, isoprene synthase gene expression, and kinetics of terminal enzymes of the MEP pathway. However, for 1,8-cineole, isoprene, β -cadinene, and α -copaene emissions, their constitutive and stress-induced *de novo* emission mechanisms are more complicated. *Eucalyptus globulus* has numerous storage glands in leaves, where terpenes are primarily stored (Külheim et al., 2015) and thus, there is a slow diffusion of volatile isoprenoids out of the storage structures (Copolovici and Niinemets, 2016; Grote and Niinemets, 2008; Kanagendran et al., 2018). In addition, a hexane extract study indicated that 1,8-cineole, isoprene, β -cadinene, and α -copaene are stored in storage glands in *E. globulus* leaves (Kanagendran et al., unpublished). Along with storage emissions, *de novo* emission of mono- and sesquiterpenes in a light- and temperature-dependent manner can contribute to the emission blend under stressed and non-stressed conditions (Grote et al., 2013; Holzke et al., 2006). In our study, the highest emission burst of 1,8-cineole and isoprene detected at 0.5 h since stress applications was most probably due to a severe damage of epithelial and epidermal cells of storage glands as a result of hypersensitive response upon ozone and wounding treatments (Bussotti et al., 2007). As we demonstrated in a previous paper, the emission of iconic LOX volatiles such as (*E*)-2-hexenal and 3-(*Z*)-hexanol further confirmed the cellular damage in foliage storage glands of *E. globulus* (Kanagendran et al. (2018) for further details). Once damaged, 1,8-cineole, isoprene, β -cadinene, and α -copaene will be emitted from the storage glands until the damaged parts are either sealed by terpenoid oxidization and polymerization or the terpene pools are exhausted (Loreto et al., 2000; Schuh et al., 1996). The emission kinetics observed in our study suggested that the storage emissions and stress-induced *de novo* emissions contributed to the total emission blend of 1,8-cineole and isoprene throughout the recovery phase.

A modified expression pattern of isoprene, 1,8-cineole, and isoprene synthase genes is expected to ultimately contribute to the total protein content of corresponding terpene synthases. However, in most of the cases, there were inverse relationship between isoprene, 1,8-cineole, and isoprene synthase gene expressions with corresponding terpene emissions rates (Fig. 2-4 and Table 3). This suggests that there was a certain time-delay between the

onset of gene expression, and biosynthesis and emission rates of volatile isoprenoids, and that the lag-time was shorter for wounding treatment than for separate ozone and combined ozone and wounding treatments. In addition, we cannot exclude the share of storage emission contributing to the total emission blend, particularly at the shorter recovery phase, responsible for the poor correlations between terpene synthase gene expression and terpenoid emissions to some extent. Therefore, further studies are required to differentiate the storage and *de novo* emissions of 1,8-cineole, isoprene, α -pinene, and α -copaene upon combined ozone and wounding treatments in *E. globulus* leaves and correlate the temporal changes in *de novo* emissions with terpene synthase gene expression profiles.

4.4 A lag-time between TPS gene expression and emission: important factors to be considered

In our study, in transient conditions after ozone and wounding treatments, the correlations between gene expression and emission responses were strong and positive, and in some cases, the correlations were negative (Fig. 2-4 and Table 3). Furthermore, we applied a time-delay analysis to study time-shifts between gene expression and emission responses by varying the time-delay from 1 h to 6 h. We observed that the best correspondence between gene expression and product emission was without any time-shift for the separate wounding treatments and with 0-2 h shift for separate ozone, and combined ozone and wounding treatments (Fig. 2-4 and Table 3).

In fact, all steps from the onset of stress application to terpene biosynthesis, including signal perception, signal transduction, activation of transcription factors, and gene expression are time-consuming. In addition, there should be a continuous supply of corresponding proteins, such as terminal enzymes in MEP and MVA pathways to get a strong positive correlation between terpene synthase gene expression and terpene emission. Indeed, there is a lack of information about the rate of protein turnover in response to different abiotic stresses. However, heat shock treatments in tomato (*Solanum lycopersicum*) (Pazouki et al., 2016) and herbivory feeding in European alder (*Alnus glutinosa*) (Copolovici et al., 2014; Copolovici et al., 2011) demonstrated *de novo* synthesis of terpenes due to altered terpene synthase expression started in a few hours after the onset of stress applications and terpene emission reached the pre-stress level within hours of removal of stress treatments. So far, there is no information about how separate and combined acute ozone and wounding treatments affect continuous turnover and enzyme kinetics of terpene synthases through recovery after stress. Nevertheless, a previous study indicated that chronic ozone exposures downregulated isoprene synthase enzyme activity and isoprene synthase gene expression in field-grown aspen trees (*Populus tremuloides* Michx.), ultimately leading to decreased isoprene emission (Calfapietra et al., 2007). Thus, in our study, the negative and poor correlations between isoprene synthase gene expression and emission were also likely associated with decreased isoprene synthase enzyme activity, associated with the downregulation of isoprene synthase gene expression (Fig. 2 and Table 3).

However, we cannot rule out the presence of other physiological and physico-chemical factors that were superimposed gene expression level control, ultimately leading to weak and negative correlations. First, kinetics of key enzymes in MEP and MVA pathways could have

been altered along with gene expression changes upon ozone and wounding treatments, leading to significant changes in terpenoid emission responses through recovery after stress; second, particularly for monoterpene 1,8-cineole and sesquiterpenes isoprene, α -pinene, and α -copaene, a significant quantity of both storage emissions due to the damage of storage structures and *de novo* emissions contributes to the total emission blend in response to ozone and wounding treatments, especially at shorter recovery phases; third, in this study, ROS produced in mesophyll cells by oxidative stress were also removed by the reaction of volatile isoprenoids through recovery phase (Loreto and Fares, 2007) and thus, a certain proportion of biosynthesized isoprene, 1,8-cineole, isoprene, α -pinene, and α -copaene was scavenged in intercellular air-spaces; fourth, although minor, the reduction of substrate concentrations, DMADP for isoprene, and GDP for 1,8-cineole, due to time-dependent reduction in photosynthesis could likely account for the declined emissions of isoprene and 1,8-cineole to some extent (Fig. S1B, and Fig. S2A and Fig. S2B) (Niinemets et al., 2002; Staudt and Lhoutellier, 2011); and fifth, the emission of 1,8-cineole could have been declined by reduced stomatal conductance for ozone and wounding treatments (Fig. S1C and Fig S2B), and the emission of isoprene could slightly have been enhanced due to positive effect of reduced intercellular CO₂ concentration on isoprene biosynthesis (Rasulov et al., 2016).

Generally, temporal changes in transcript abundance of isoprene, 1,8-cineole, and isoprene synthase genes demonstrated antagonistic effects (Fig. 1A-C), while the emission responses showed synergistic effect upon combined ozone and wounding treatments throughout the recovery phase (Fig. S2A-C). Therefore, future research is warranted to understand the complexity of isoprenoid emissions upon different stress applications, involving reciprocal and inverse relationships between terpene synthase gene expression profiles and rate limiting terminal enzyme kinetics, ultimately responsible for *de novo* emission and factors affecting storage emissions.

4.5 A lag-time between stomatal ozone uptake rate and terpene synthase gene expression: factors affecting the relationship

Stomata are the primary passage through which ozone enters leaf intercellular airspaces and alters gene expression profile (Beauchamp et al., 2005; Heath, 2008). In most of the cases, the expression of terpene synthase genes showed an inverse relationship with stomatal ozone uptake rates through recovery phase for both separate ozone treatments, and combined ozone and wounding treatments (Fig. 5-7 and Table 4). Once entered the intercellular air-spaces, the oxidative potential of ozone depends on the extent to which ozone is scavenged by water-soluble antioxidants such as putrescine and apoplastic ascorbate within the aqueous phase in cell walls and by volatile antioxidants such as isoprene, mono- and sesquiterpenes within the leaf gas phase as well as by lipid-soluble volatile (Bouvier-Brown et al., 2009; Fares et al., 2010; Goldstein et al., 2004; Kurpius and Goldstein, 2003). In addition, there is also a certain delay between ozone uptake and physiological responses, including signal transduction, activation of transcription factors and eventually, the onset of changes in terpene synthase gene expression profiles. Furthermore, the biosynthesis of non-volatile antioxidants can scale with the quantity of stomatal ozone flux (Booker and Fiscus, 2005). Taken together, our data suggest that negative and poor correlations between stomatal ozone

uptake rates and terpene synthase gene expressions was likely due to enhanced removal of ozone in leaf cells and the time-delay for the elicitation of terpene synthase gene expression. However, we cannot rule out the impact of stomatal ozone uptake on the regulation of pertinent transcription factors. Thus, a detail understanding of the effects of stomatal ozone uptake on the regulation of transcription factors for terpene synthase genes deserves an in-depth study.

5 Conclusions

We investigated the time-courses of expression profiles of isoprene, 1,8-cineole, and isoprene synthase genes in response to ozone and wounding treatments and their relationship with stomatal ozone uptake and emission rates. Collectively, these data indicate that both temporal changes in isoprene, 1,8-cineole and isoprene emissions and changes in photosynthetic characteristics were synergistically altered, but the expression profiles of isoprene, 1,8-cineole and isoprene synthase genes were antagonistically altered by combined stress treatments. However, the synergistic effect did not scale with the severity of stress applications. Overall, the relationship between the expression of terpene synthase genes and emission rates upon ozone and wounding treatments varied with the type of stress application and localization of terpene synthase enzymatic activity in plant cells. The strong positive correlations between terpene synthase gene expression and emission indicated that the lag-time between the gene expression and biosynthesis of three volatile isoprenoids was shorter and that the biosynthesis was primarily controlled at gene-level, and weak and negative correlations indicated that the lag-time was longer, indicative of lower rate of terpene synthase turnover after stress. Thus, in these conditions, the gene-expression level control was insignificant. The time-delay analysis demonstrated that there was no time-shift between the emission and gene expression in the wounding treatment for 1,8-cineole and isoprene, indicative of higher protein turn-over within shorter recovery phase after stress applications and / or the significant contribution of storage emissions to the total emission blend, particularly at shorter recovery phase. However, the relationship between time-dependent changes in gene expression and emission responses was stronger for combined ozone and wounding treatments, followed by separate ozone and wounding treatments. In addition, the inverse relationship between stomatal ozone uptake and gene expression was likely due to a lag-time between ozone uptake and the onset of gene expression and also due to enhanced removal of ozone in the leaf by volatile and non-volatile antioxidants.

Overall, the three-way interactions among ozone uptake, gene expression, and product emission responses in this study underscore the complexity of relationship between terpene synthase gene expression and terpenoid emissions for different type and degree of oxidative stresses. However, further studies related to temporal regulation of terpene synthase genes and subsequent turnover of gene products, and differential regulation of terpene synthase enzyme kinetics, pertinent transcription factors, and their stress-stimulus specific mechanism of action are highly warranted to gain insight into temporal kinetics of terpene synthase gene expression and emission responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

VOCs	volatile organic compounds
Eucons04	cyclin-dependent kinase E-1
Eucons08	transcription elongation factor S-II
GLM	generalized linear model
LOX volatiles	lipoxygenase pathway volatiles
MEP/DOXP pathway	2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate pathway
MVA pathway	mevalonate pathway
PSII	photosystem II
qPCR	quantitative polymerase chain reaction
ROS	reactive oxygen species
TPS	terpene synthase

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Fig. 1.

Relative expression levels (means + SE) of isoprene (A), 1,8-cineole (B), and isoprene synthase (C) genes in non-treated control leaves, wounded, ozone-exposed (4, 5, and 6 ppm ozone for 3 h), and ozone-exposed and wounded (O_3 + W) (first exposed to 4, 5, and 6 ppm ozone for 3 h and then wounded) leaves of *E. globulus* at different recovery times (0.5, 3, 10, 25, 50, and 75 h) after stress applications. The selected leaves were exposed to ozone for 3 hours at given ozone concentration, and wounding stress was applied by punching four (area of each leaf disc was 25 mm²) holes in the leaf lamina by a paper punch for a total perimeter

length (wound edge) of 7 cm per leaf. In this experiment, 1-year old and ca. 1 m tall plants with similar biomass, and stem thickness were used. Gas exchange measurements were conducted under baseline conditions in the glass chamber as follows: photosynthetic photon flux density at the leaf surface of 700 - 750 $\mu\text{mol m}^{-2} \text{s}^{-1}$, leaf temperature of 25 - 27 °C, ambient CO_2 concentration of 380-400 $\mu\text{mol mol}^{-1}$, and relative air humidity of 60 - 70 %. All measurements were replicated at least thrice. Individual effects of ozone, wounding, and recovery time (Time), and their interactions on relative gene expression levels were tested by generalized linear models (GLM) with maximum likelihood model fitting (Table 2 for the statistical analysis).

Fig. 2.

Relationships between relative gene expression and emission rate of isoprene in wounded, ozone-exposed (4, 5, and 6 ppm ozone for 3 h), and ozone-exposed and wounded (O₃ + W) (first exposed to 4, 5, and 6 ppm ozone for 3 h and then wounded) leaves of *E. globulus* at different recovery times (0.5, 3, 10, and 25 h) after stress application (stress application protocol as in Fig. 1, gene expression data from Fig. 1A, and emission data from Fig. S2A). Data were fitted by linear regressions.

Fig. 3.

Relationships between relative gene expression and emission rate of 1,8-cineole in wounded, ozone-exposed (4, 5, and 6 ppm ozone for 3 h), and ozone-exposed and wounded (O₃ + W) (first exposed to 4, 5, and 6 ppm ozone for 3 h and then wounded) leaves of *E. globulus* at different recovery times (0.5, 3, 10, and 25 h) after stress application (stress application protocol as in Fig. 1, gene expression data from Fig. 1B, and emission data from Fig. S2B). Data were fitted by linear regressions.

Fig. 4.

Relationships between relative gene expression levels of isoleudene synthase and total emission rates of isoleudene synthase products (isoleudene, -cadinene, and α -copaene) in wounded, ozone-exposed (4, 5, and 6 ppm ozone for 3 h), and ozone-exposed and wounded (O₃ + W) (first exposed to 4, 5, and 6 ppm ozone for 3 h and then wounded) leaves of *E. globulus* at different recovery times (0.5, 3, 10, and 25 h) after stress application (stress application protocol as in Fig. 1, gene expression data from Fig. 2C, and isoleudene emission data from Fig. S2C). Data were fitted by linear regressions.

Fig. 5.

Dependencies of relative gene expression levels of isoprene synthase on stomatal ozone uptake rate through different recovery times (0.5, 3, 10, and 25 h) in *E. globulus* leaves after the leaves were treated with ozone for 3 h (4, 5, and 6 ppm ozone) and combined ozone and wounding treatments (first exposed to 4, 5, and 6 ppm ozone for 3 h and then wounding). Data were fitted by linear regressions.

Fig. 6.

Relative gene expression level of 1,8-cineole synthase in relation to stomatal ozone uptake rate through different recovery times (0.5, 3, 10, and 25 h) in *E. globulus* leaves after the leaves were treated with ozone for 3 h (4, 5, and 6 ppm ozone) and combined ozone and wounding treatments (first exposed to 4, 5, and 6 ppm ozone for 3 h and then wounding). Data were fitted by linear regressions.

Fig. 7.

Correlations of relative gene expression levels of isoleudene synthase with stomatal ozone uptake rate through different recovery times (0.5, 3, 10, and 25 h) in *E. globulus* leaves after the leaves were treated with ozone for 3 h (4, 5, and 6 ppm ozone) and combined ozone and wounding treatments (first exposed to 4, 5, and 6 ppm ozone for 3 h and then wounding). Data were fitted by linear regressions.

Table 1

Primer sequences for isoprene synthase, monoterpene 1,8-cineole synthase, and sesquiterpene isodene synthase used in this study

Gene	Primer sequences (5' → 3')	Amplicon length (bp)
Reference genes		
Eucons04	TACAAGCGCTGTTGATATGTGGGC / TTGCCAATGAGGCGGATTCACAAG	196
Eucons08	TCCAATCCGAGTCGCTGTCATTGT / TGATGAGCCTCTCTGGTTTGACCT	152
Target genes		
Isoprene synthase	AAGCATGAGAAAGGAGGAGC / GATATCATTGGTAAGCCGGA	91
1,8-Cineole synthase	AGCAAGCCCAAGCATAAGAAAG / CGATGAGGAGGCGATATCAT	112
Isodene synthase	TTGATGCCATGGATGGACTA / CATAGGCAAGGCGGTATGAT	120

Reference gene (cyclin-dependent kinase E-1 (*Eucon04*, Genbank accession number EEF43392.1) and transcription elongation factor s-II (*Eucon08*, Genbank accession number EEF33688.1) primers were used as described by de Oliveira *et al.* (2012). Primers for isoprene synthase gene (Genbank accession number XM_010037321.2), 1,8-cineole synthase gene (Genbank accession number DD464633.1), and isodene synthase genes (Külheim *et al.*, 2015) were designed using Primer3Plus.

Table 2

Results of generalized linear model for individual and interactive effects of ozone, wounding, ozone and wounding, and recovery time on relative expression levels of isoprene, 1,8-cineole, and isoprene synthase genes in *E. globulus* leaves. Stress application protocol as in Fig. 1.

Effect	Isoprene synthase gene expression			1,8-Cineole synthase gene expression			Isoprene synthase gene expression		
	χ^2	df	P value	χ^2	df	P value	χ^2	df	P value
Ozone	38.245	1	<0.001***	57.784	1	<0.001***	3.443	1	0.064
Wounding	29.433	1	<0.001***	25.286	1	<0.001***	47.154	1	<0.001***
Ozone + Wounding	30.874	1	<0.001***	84.561	1	<0.001***	1.206	1	0.272
Time	38.172	1	<0.001***	31.693	1	<0.001***	0.694	1	0.405
Ozone x Ozone	14.042	1	<0.001***	0.597	1	0.440	22.449	1	<0.001***
(Ozone + Wounding) x (Ozone + Wounding)	0.001	1	0.993	6.348	1	0.012*	9.840	1	0.002**
Time x Time	38.553	1	<0.001***	32.459	1	<0.001***	0.432	1	0.511
Ozone x Time	15.155	1	<0.001***	13.346	1	<0.001***	2.878	1	0.090
(Ozone + Wounding) x Time	3.963	1	0.047*	4.026	1	0.045*	0.078	1	0.780
Wounding x Time	2.798	1	0.094	2.178	1	0.140	3.877	1	0.049

Table 3

Time-delay analysis of the relationship between the relative gene expression levels and emissions of isoprene, 1,8-cineole, and isodene.

Treatment	Time-shift in gene expression (h)	Regression statistics					
		Isoprene		1,8-Cineole		Isodene	
		<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Wounding	1	0.277	0.181	0.558	0.004**	0.246	0.235
	2	0.230	0.157	0.377	0.068	0.006	0.975
	3	0.245	0.259	0.270	0.213	0.025	0.911
	4	0.187	0.404	0.192	0.391	0.053	0.814
	5	0.133	0.564	0.128	0.580	0.075	0.745
	6	0.088	0.712	0.066	0.783	0.076	0.750
4 ppm ozone	1	0.465	0.019*	0.600	0.002**	0.698	<0.001***
	2	0.392	0.058	0.222	0.296	0.611	0.002**
	3	0.337	0.116	0.130	0.557	0.479	0.020*
	4	0.028	0.902	0.374	0.086	0.338	0.123
	5	0.216	0.347	0.371	0.097	0.189	0.412
	6	0.330	0.196	0.386	0.092	0.029	0.904
5 ppm ozone	1	0.346	0.090	0.247	0.234	0.925	<0.001***
	2	0.218	0.305	0.329	0.116	0.849	<0.001***
	3	0.175	0.425	0.429	0.041*	0.751	<0.001***
	4	0.113	0.616	0.551	0.008**	0.639	0.001**
	5	0.048	0.834	0.690	<0.001***	0.516	0.016**
	6	0.001	0.991	0.838	<0.001***	0.384	0.094
6 ppm ozone	1	0.750	<0.001***	0.351	0.085	0.511	0.009**
	2	0.776	<0.001***	0.490	0.015*	0.522	0.009**
	3	0.292	0.175	0.596	0.003**	0.566	0.005**
	4	0.870	<0.001***	0.685	<0.001***	0.600	0.003**
	5	0.869	<0.001***	0.769	<0.001***	0.627	0.002**
	6	0.831	<0.001***	0.846	<0.001***	0.647	0.002**
4 ppm ozone + wounding	1	0.690	<0.001***	0.869	<0.001***	0.854	<0.001***
	2	0.630	<0.001***	0.713	<0.001***	0.628	<0.001***
	3	0.514	0.012*	0.711	<0.001***	0.496	0.016*
	4	0.382	0.080	0.710	<0.001***	0.362	0.097
	5	0.290	0.230	0.710	<0.001***	0.232	0.312
	6	0.093	0.697	0.710	<0.001***	0.114	0.633
5 ppm ozone + wounding	1	0.675	<0.001***	0.822	<0.001***	0.432	0.031*

Treatment	Time-shift in gene expression (h)		Regression statistics				
6 ppm ozone + wounding	2	0.570	0.003**	0.603	0.002**	0.394	0.057
	3	0.480	0.020*	0.461	0.027*	0.271	0.209
	4	0.390	0.072	0.297	0.180	0.142	0.528
	5	0.301	0.186	0.113	0.625	0.003	0.988
	6	0.212	0.368	0.087	0.716	0.145	0.542
	1	0.458	0.021*	0.522	0.007**	0.174	0.405
	2	0.514	0.010**	0.280	0.185	0.297	0.157
	3	0.593	0.003**	0.275	0.203	0.245	0.260
	4	0.674	<0.001***	0.271	0.222	0.189	0.399
	5	0.757	<0.001***	0.274	0.229	0.124	0.592
	6	0.833	<0.001***	0.295	0.206	0.035	0.882

Both relative gene expression levels and isoprenoid emissions were measured at the same time after separate and combined ozone and wounding treatments and then time-shifts of 1–6 h were introduced in the gene expression data to consider the circumstance that a change in gene expression comes first and emission response follows with a certain time delay. After each time-shift, data were fitted by linear regressions. r is the correlation coefficient P is the statistical probability that $r = 0$.

Table 4

Time-delay analysis of the relationship between stomatal ozone uptake rates and the relative gene expression levels of isoprene synthase, 1,8-cineole synthase, and isoleudene synthase

Treatments	Time shift in ozone uptake (h)	Regression statistics					
		Isoprene		1,8-Cineole		Isoledene	
		<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
<hr/>							
4 ppm ozone	1	0.006	0.978	0.886	<0.001***	0.523	0.008**
	2	0.173	0.429	0.686	<0.001***	0.261	0.228
	3	0.215	0.337	0.685	<0.001***	0.252	0.257
	4	0.267	0.242	0.683	<0.001***	0.244	0.285
	5	0.329	0.157	0.683	<0.001***	0.245	0.297
	6	0.396	0.093	0.685	0.001**	0.268	0.267
5 ppm ozone	1	0.248	0.241	0.467	0.021*	0.803	<0.001***
	2	0.292	0.176	0.286	0.186	0.823	<0.001***
	3	0.236	0.290	0.319	0.147	0.821	<0.001***
	4	0.202	0.378	0.355	0.114	0.822	<0.001***
	5	0.182	0.442	0.394	0.085	0.832	<0.001***
	6	0.587	0.010*	0.440	0.059	0.848	<0.001***
6 ppm ozone	1	0.814	<0.001***	0.821	<0.001***	0.708	<0.001***
	2	0.680	<0.001***	0.700	<0.001***	0.635	0.001**
	3	0.688	<0.001***	0.709	<0.001***	0.674	<0.001***
	4	0.684	<0.001***	0.709	<0.001***	0.690	<0.001***
	5	0.679	<0.001***	0.707	<0.001***	0.692	<0.001***
	6	0.677	0.001**	0.705	<0.001***	0.698	<0.001***
4 ppm ozone + wounding	1	0.710	<0.001***	0.672	<0.001***	0.858	<0.001***
	2	0.603	0.002**	0.552	0.006**	0.595	0.003**
	3	0.561	0.006**	0.503	0.016*	0.429	0.046*
	4	0.510	0.018*	0.457	0.036*	0.258	0.258
	5	0.460	0.041*	0.418	0.066	0.086	0.718
	6	0.423	0.071	0.390	0.098	0.077	0.752
5 ppm ozone + wounding	1	0.609	<0.001***	0.073	0.736	0.617	0.001**
	2	0.665	<0.001***	0.115	0.600	0.674	<0.001***
	3	0.706	<0.001***	0.121	0.591	0.719	<0.001***
	4	0.758	<0.001***	0.126	0.587	0.770	<0.001***
	5	0.823	<0.001***	0.122	0.606	0.830	<0.001***
	6	0.898	<0.001***	0.097	0.694	0.892	<0.001***
6 ppm ozone + wounding	1	0.542	0.006**	0.709	<0.001***	0.960	<0.001***

Treatments	Time shift in ozone uptake (h)			Regression statistics			
	2	0.352	0.098	0.542	0.007**	0.892	<0.001***
	3	0.367	0.092	0.545	0.008**	0.871	<0.001***
	4	0.380	0.089	0.548	0.010*	0.849	<0.001***
	5	0.394	0.085	0.550	0.012*	0.827	<0.001***
	6	0.408	0.083	0.553	0.014	0.798	<0.001***

Both stomatal ozone uptake rates and relative gene expression levels were measured at the same time after separate ozone and combined ozone and wounding treatments. Thereafter, time-shifts of 1–6 h in ozone uptake were introduced in the ozone uptake data to consider the circumstance that a change in ozone uptake data comes first and gene expression follows with a certain time delay. After each time-shift, data were fitted by linear regressions. r is the correlation coefficient for the cross correlation and P is the statistical probability.