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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 isoledene 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 isoledene 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 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 isoledene synthase genes differentially influenced their corresponding product emissions for separate and combined ozone and wounding treatments through recovery.

1. Introduction

Ozone is a key air pollutant and a plant stress elicitor. It is mainly formed in the troposphere when nitrogen oxides (NOx) 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, 2010). 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, 2010). 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

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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in key metabolic activities such as photosynthesis and plant growth, ozone exposure leads to substantial alternations 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, 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 hard-wood 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 isoledene synthase (TPS-a). We demonstrated that there was a great variation of emission responses of isoprene, 1,8-cineole, and isoledene 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 isoledene, (2) relate the expression profiles of isoprene, 1,8-cineole, and isoledene synthase genes with product emissions through recovery, (3) investigate the time-delay correlation between isoprene, 1,8-cineole, and isoledene 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 isoledene 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 isoledene 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 isoledene 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 µmol m^{-2} s^{-1} with 12 h photoperiod, chamber temperature (day/night) of 28/25 °C, relative humidity of 60–70 %, and ambient CO_2 concentration of 380–400 µmol 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