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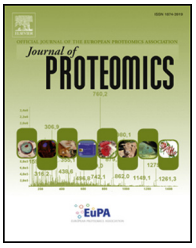

Large-scale protein analysis of European beech trees following four vegetation periods of twice ambient ozone exposure

Q1 René Kerner[a,b,*], Edgar Delgado-Eckert[c], Dieter Ernst[b], Jean-William Dupuy[d], E.E. Thorsten Grams[e], J. Barbro Winkler[f], Christian Lindermayr[b], Gerhard Müller-Starck[a]

[a]Technische Universität München, Section of Forest Genetics, Hans-Carl-von-Carlowitz-Platz 2, 85354 Freising, Germany
[b]Helmholtz Zentrum München, Institute of Biochemical Plant Pathology, Ingolstädter Landstraße 1, 85764 Neuherberg, Germany
[c]University Children's Hospital (UKBB) at University of Basel, Spitalstrasse 33, 4031 Basel, Switzerland
[d]Université de Bordeaux, Centre de Génomique Fonctionnelle, Plateforme Protéome Bordeaux, 33000 Bordeaux, France
[e]Technische Universität München, Ecophysiology of Plants, Department of Ecology and Ecosystem Sciences, Hans-Carl-von-Carlowitz-Platz 2, 85354 Freising, Germany
[f]Helmholtz Zentrum München, Institute of Biochemical Plant Pathology Research Unit Environmental Simulation, Ingolstädter Landstraße 1, 85764 Neuherberg, Germany

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ABSTRACT

In the present study, we performed a large-scale protein analysis based on 2-DE DIGE to examine the effects of ozone on the leaves of juvenile European beech (*Fagus sylvatica* L.), one of the most important deciduous trees in central Europe. To this end, beech trees were grown under field conditions and subjected to ambient and twice ambient ozone concentrations during the vegetation periods of four consecutive years. The twice ambient ozone concentration altered the abundance of 237 protein spots, which showed relative ratios higher than 30% compared to the ambient control trees. A total of 74 protein spots were subjected to mass spectrometry identification (LC–MS/MS), followed by homology-driven searches. The differentially expressed proteins participate in key biological processes including the Calvin cycle and photosynthesis, carbon metabolism, defense- and stress-related responses, detoxification mechanisms, protein folding and degradation, and mechanisms involved in senescence. The ozone-induced responses provide evidence of a changing carbon metabolism and counteraction against increased levels of reactive oxygen species.

Biological significance
This study provides useful information on how European beech, an economically and ecologically important tree, reacts on the molecular level to increased ozone concentrations

Abbreviations: 2ME, 2-mercaptoethanol; ACN, acetonitrile; ADK, adenosine kinase 2; Aldolase, fructose bisphosphate aldolase; GalUR reductase, galacturonic acid reductase; GS1, glutamine synthetase; GSH, glutathione; JA, jasmonic acid; PEP, phosphoenolpyruvate; PHGDH, D-3-phosphoglycerate dehydrogenase; PPO, polyphenol oxidase; PRK, phosphoribulokinase; Probable GST, probable glutathione S-transferase; ROS, reactive oxygen species; RuBisCO, ribulose-1,5-bisphosphate-carboxylase/-oxygenase; SA, salicylic acid; SAM, S-adenosyl-methionine; SBP1, selenium-binding protein 1; SBPase, sedoheptulose 1,7 bisphosphatase; TKL, transketolase.

* *Corresponding author at:* Helmholtz Zentrum München, Institute of Soil Ecology, Ingolstädter Landstraße 1, 85764 Neuherberg, Germany. Tel.: +49 89 3187 4046; fax: +49 89 3187 2800.
E-mail address: renekerner@mytum.de (R. Kerner).

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expected in the near future. The main emphasis in the present study was placed on identifying differentially abundant proteins after long-term ozone exposure under climatically realistic settings, rather than short-term responses or reactions under laboratory conditions. Additionally, using nursery-grown beech trees, we took into account the natural genotypic variation of this species. As such, the results presented here provide information on molecular responses to ozone in an experimental plant system at very close to natural conditions. Furthermore, this proteomic approach was supported by previous studies on the present experiment. Ultimately, the combination of this proteomic approach with several approaches including transcriptomics, analysis of non-structural carbohydrates, and morphological effects contributes to a more global picture of how beech trees react under increased ozone concentrations.

1. Introduction

Tropospheric ozone is formed through the reaction of anthropogenically produced air pollutants such as nitrogen oxides (NOx), hydrocarbons and volatile organic compounds (VOCs) in the presence of sunlight. Since the beginning of industrial development, global ozone concentrations have risen and are predicted to further increase at a global scale and to persist at relatively high levels in central Europe [1,2]. Due to its powerful oxidizing properties and, consequently, its ability to damage organic molecules, ozone has been well established to be detrimental for living organisms. For plants, ozone is considered to be one of the most toxic air pollutants, and it is regarded as a risk factor for forest trees [3,4]. The type and severity of the reaction can vary depending on the concentration, weather conditions, the duration of exposure and the age and genetic predisposition of plants. It is estimated that tropospheric ozone is responsible for 10% of the reduction of the European forest crop yield [5]. Several factors that may explain this effect are decreases in gas exchange [6], the carboxylation deficiency, and net photosynthesis [7–10]. At the molecular level, these reactions are partially explained by reduced protein activities/amounts of the carbon fixation molecule RuBisCO (ribulose-1,5-bisphosphate-carboxylase/-oxygenase), the related enzyme RuBisCO activase, and photosystem II-associated proteins [7,8,10]. As a consequence of reduced CO_2 fixation, a smaller quantity of triose phosphate molecules is exported from the chloroplast. Therefore, plants may activate catabolic pathways such as glycolysis, the pentose phosphate pathway, and mitochondrial respiration to feed the Krebs cycle with carbon skeletons [7,10–12]. Furthermore, ozone decomposes rapidly in the apoplast of leaves. Its destruction is followed by the formation of superoxide radicals ($O_2.^-$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH.^-$), which consequently triggers a cellular oxidative burst [13,14]. Increased concentrations of apoplastic reactive oxygen species (ROS) beyond a threshold induce changes in the guard cells, thereby propagating secondary endogenous ROS accumulation and activation of mitogen-activated protein kinase (MAPK) [15]. MAPK activation, in turn, appears to be involved in the increased synthesis of ethylene (ET) which, together with salicylic acid (SA), results in the death of affected cells and the formation of local lesions. In contrast to this mechanism, jasmonic acid (JA) acts antagonistically to contain the spread of cell death [16,17]. Depending on the fine tuning of these counteracting compounds, plants will induce either cell death or the production of defense signals such as phenolics, phytoalexins, and pathogenesis-related (PR) proteins.

Proteomics offers great potential to obtain a more global picture of cell responses in organisms subjected to different environmental conditions. This technology is gaining increased popularity in non-model species as the genomic data of such organisms are becoming available [18]. In the case of European beech, the recent availability of 37,632 ESTs (http://www.evoltree.com) and 200,402 ESTs for the taxon Fagaceae (http://www.ncbi.nlm.nih.gov/) facilitates the large-scale analysis of gene functions. Although proteomics studies have been conducted in forest trees [18], there is limited information regarding the differential modulation of proteins in woody plants after long-term ozone exposure under a set of ecologically realistic conditions. Therefore, the main emphasis in the present study was placed on identifying differentially abundant proteins after long-term ozone exposure under climatically realistic settings, rather than short-term responses or reactions under laboratory conditions. Additionally, using nursery-grown beech trees, we took into account the natural genotypic variation of this species. As such, the results presented here provide information on molecular responses to ozone in an experimental plant system at very close to natural conditions.

2. Materials and methods

2.1. Experimental design and exposure to free-air ozone fumigation

The experiment was conducted during a period of 7 years at the outdoor lysimeter facilities of the Helmholtz Zentrum München, Germany (48°13′ N 11°36′ E, 490 m altitude). The trial involved eight lysimeters and a surrounding area. Plants grown in four lysimeters and in the adjacent areas were exposed to a twice ambient ozone concentration (treatments), while the other half (controls) were exposed to ambient ozone fumigation (Fig. 1A). A total of 20 beech trees surrounding the lysimeters (n = 10 per group) were exclusively used for measurements for the proteome analysis presented herein and a transcript analysis presented elsewhere [19]. Moreover, beech trees grown in lysimeters (n = 16 per group) were used in 2006 for measurements of plant growth and biomass [20] as well as for an analysis of non-structural carbohydrates [21]. Briefly, soil from the "Höglwald" forest site was used to fill the lysimeters and the surrounding area in 1999. For the subsequent 3 years, the soil was left untreated to ensure the development of a representative soil structure. In November

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