

Biological significance

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

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1. Introduction

Tropospheric ozone is formed through the reaction of anthropogenically produced air pollutants such as nitrogen oxides (NO_x), 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

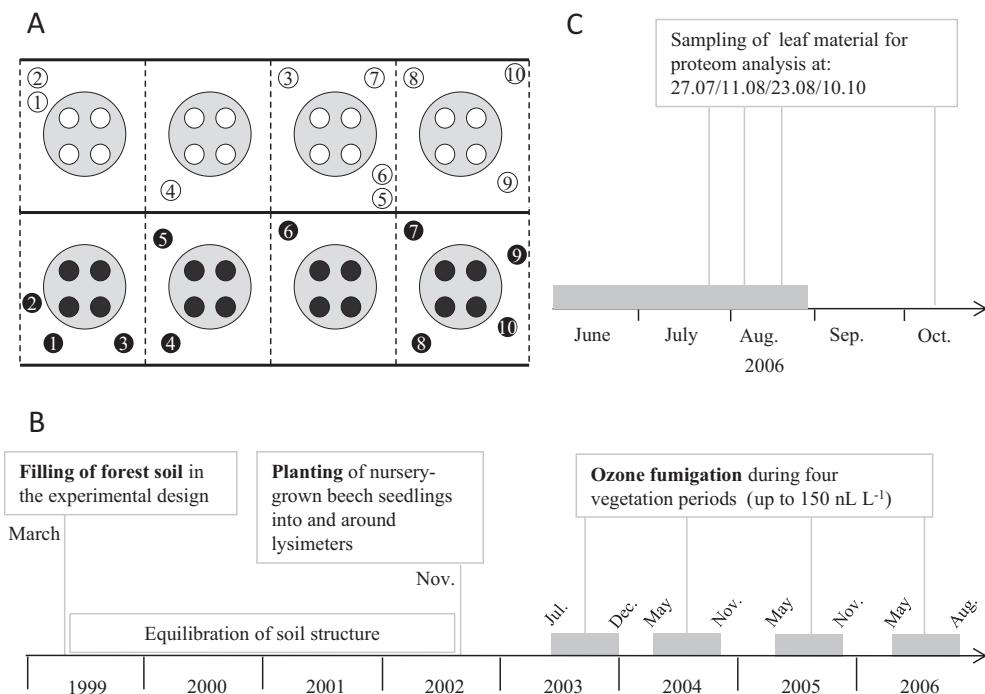


Fig. 1 – A: Schematic sketch of the lysimeter experiment. Each lysimeter – represented as a gray circle – consisted of four juvenile beech trees. White and black circles indicate juvenile beech trees fumigated with ambient ozone and twice ambient ozone, respectively. The numbered beech trees around the lysimeters were used for proteomic analysis. **B:** Schematic representation of the lysimeter experiment with the time line. Gray blocks indicate the fumigation time during each year. **C:** Timeline for the evaluation of visually detectable ozone damage (dashed line; n = 4 trees/group and time point) and sampling time points for protein analysis (continuous line; n = 10 trees/group and time point).

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 2002, four nursery-grown juvenile European beech trees (three-years old and approximately 60 cm high) were planted in each lysimeter. Furthermore, beech trees of the same age were planted in the area surrounding the lysimeters (4 plants per m²) to provide a homogeneous stand. Maintenance of lysimeters comprised removal of weeds and control of occurrence of pests. Twice ambient free-air ozone fumigation started in July 2003 and ended after four vegetation periods with plant harvest in 2006. Ozone was fumigated during the day. In the first vegetation period fumigation was stopped in December 2003. For the years 2004 and 2005, ozone was fumigated from May (before bud break) until the end of October (after leaf senescence). During the year 2006, juvenile trees were fumigated from May until the end of August. The twice ambient ozone concentration was restricted to 150 nL L⁻¹ to avoid acute injury to the leaves. For the period between the beginning of April and the end of October 2006, the AOT 40 value (accumulated seasonal exposure over 40 nL L⁻¹) of the twice ambient ozone fumigation was

52.6 $\mu\text{L L}^{-1}$ h, which was 3.7 times higher than the AOT40 value in the ambient ozone lysimeters (14.3 $\mu\text{L L}^{-1}$ h). For the same period of time, the sum0 (cumulative seasonal exposure during daylight) was also higher (178.7 $\mu\text{L L}^{-1}$ h) in the enriched ozone lysimeters compared to those fumigated at ambient levels (125.1 $\mu\text{L L}^{-1}$ h) [22]. As the evapotranspiration rates were comparable for trees in the two treatments, the higher ozone concentration in the twice ambient ozone-exposed lysimeters reflected the increased simulated effective ozone influx into plants in lysimeters exposed to twice ambient ozone fumigation compared to plants exposed to ambient ozone fumigation [23]. Fig. 1 provides an overview of the experimental setup. A detailed description of the experimental design, including the free-air ozone exposure devices, can be found elsewhere [22].

2.2. Large-scale protein analysis

2.2.1. Harvesting plant tissue

For proteome analysis, 20 juvenile beech trees from the area surrounding the lysimeters were used, including 10 trees exposed to the ambient ozone concentration (controls) and another 10 trees that were subjected to twice ambient ozone fumigation. Each biological sample consisted of a pool of three leaves per tree. Leaves were collected on the 27th of July, 11th of August and 23rd of August in 2006 and 43 days after stopping ozone fumigation on 10th of October 2006. All leaves were harvested at 9:00 am MET, immediately frozen in liquid nitrogen and stored at -80 °C.

statistical analysis. For the 27th of July 2006 sampling time point, under at least two different normalization methods, 87 protein spots were found to be differentially abundant in the twice ambient ozone-exposed leaves compared to the controls (Supplementary table). A similar statement can be made for approximately 70 protein spots for the sampling time point of the 27th of July 2006 and for 100 protein spots for the sampling time point of the 23rd of August 2006. In contrast, following 43 days of recovery after twice ambient ozone exposure, October 10th 2006, the beech leaves showed only one spot that was significantly different under one normalization method compared to the control group.

The heat map representation of protein abundances and hierarchical clustering performed on the abundance profiles (Fig. 2) indicated common spots that showed statistically significant differences during July and August. With the exception of four treatment samples that clustered closer to the control group (T1_1108, T1_2308, T1_2707, T8_2707), hierarchical clustering clearly revealed differences between the control and treated groups. However, samples from different harvested time points clustered together within the same sub-branches, showing no general difference in the modulation of proteins over the three time points.

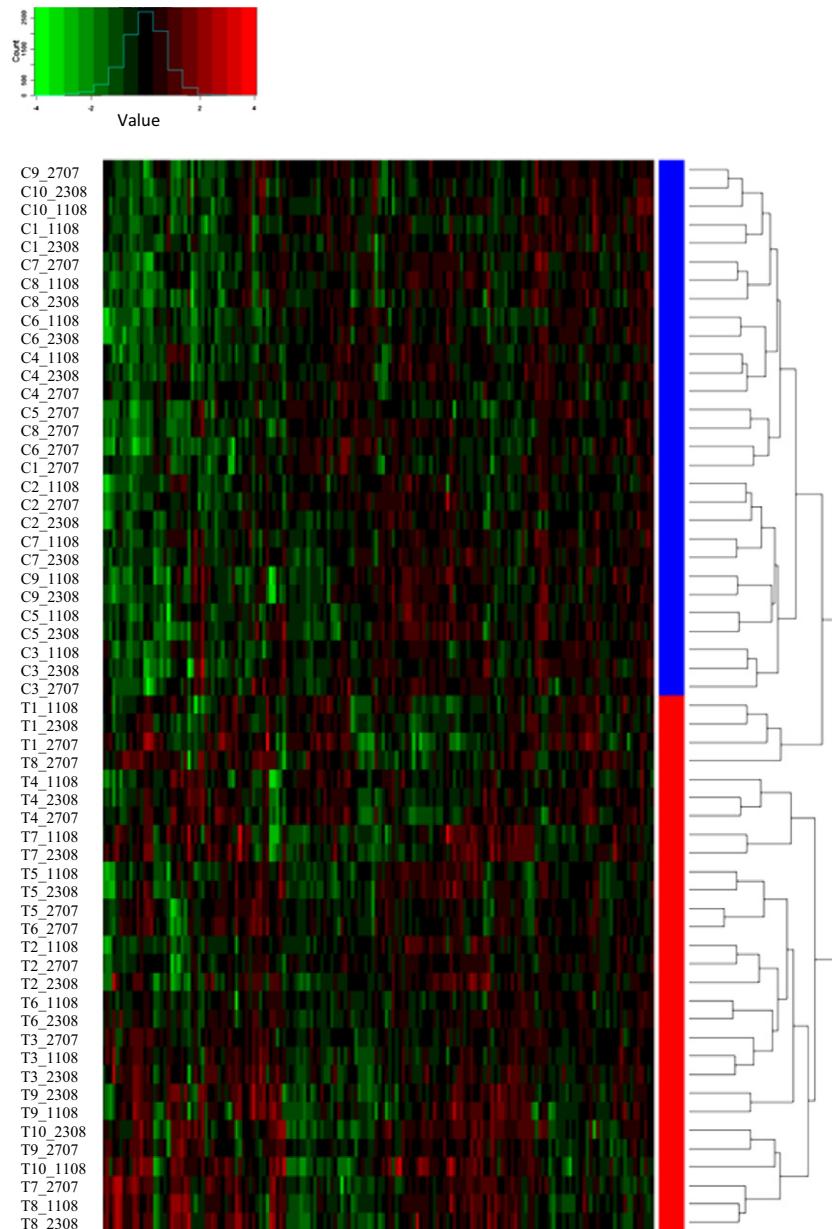


Fig. 2 – Heat map representation of protein abundances and hierarchical clustering of the abundance profiles displaying identical protein spots modulated on the 27th of July and 11th and 23rd of August 2006. The presented values are loess normalized \log_2 ratios. Green: down-regulation; Red: up-regulation. Dendrogram on the right-hand side: Data from control and treated samples are clustered based on the distance between their profiles of abundances. The color bar at the right (blue or red) indicates samples treated with ambient (blue) and twice ambient (red) ozone fumigation. Samples labeled on the left with “C” and “T” indicate controls and treatments, respectively.

Among all of the differentially abundant spots, a total of 65 resembled the preparative gel spot pattern. For subsequent protein identification, we also took into account 9 differentially abundant spots presenting significant differences under one normalization method that showed a response to ozone treatment in the previous analysis. These 74 spots were subjected to LC-MS/MS followed by a homology-driven search. The mass spectra of 2 spots failed to show any peaks, while those of 26 spots indicated mixtures of multiple protein. For the remaining 46 spots, a single protein was identified (Table 1). Based on biological functions, these proteins were classified into eleven groups: 1) Calvin cycle, 2) photosynthesis, 3) additional chloroplast proteins, 4) mitochondrial electron transport chain, 5) carbon metabolism, 6) nitrogen metabolism, 7) stress response, 8) defense response, 9) detoxification, 10) degradation and 11) protein folding (Fig. 3). The most affected group upon twice ambient ozone was the Calvin cycle, followed by proteins related to the defense mechanism and detoxification-related proteins.

The statistical information about the identified spots representing single proteins is summarized in Table 1, whereas the appearance of modulated spots in the gel is illustrated in sheet #2 of the Supplementary data. Differences between the total amount of regulated protein spots and the number of identified proteins are due to 1) the presence of multiple proteins for a specific spot, 2) the occurrence of a protein in multiple locations on the 2-DE gel and 3) the lack of information for several spots because they were visualized in the analytical CyDye gels, but not in the preparative gels.

3.3. Comparative analysis of differentially abundant transcripts and proteins during ozone treatment

We compared all of the differentially abundant proteins showing significantly differentially abundant transcripts that were previously observed to be modulated in juvenile beech trees during twice ambient ozone fumigation [19]. This comparison can be used as a global source of information to better understand changes in molecular pathways following ozone treatment in beech leaves. When the resulting data were compared at the functional classification level (corresponding to transcripts/proteins that are commonly modulated in specific molecular pathways), correlations were obtained (Fig. 4). Such correlations were observed particularly for chloroplast and photosynthesis-related molecules as well as for molecules related to disease/defense responses and detoxification mechanisms. Although the comparative analysis employed the same sample source and harvesting time points, none of the identified differentially abundant proteins showed direct overlap with the expressed genes (Fig. 4 and sheet #8 of the Supplementary data).

4. Discussion

Given that technical reproducibility has been reported to be an issue in previous studies using 2-DE gel electrophoresis [31], for the quantitative group analysis of controls versus treatments, we selected only “consistent spots”, showing low

variances regarding protein abundances among technical triplicates. Although the use of spectrally resolvable fluorescent dyes (Cy2, Cy3, and Cy5) has been reported to decrease experimental gel-to-gel variation [32], other sources of technical variation arise during the extraction, labeling and loading of proteins that must be considered when designing an experiment [33]. Overlooking these sources of variation may easily obscure the biological changes being investigated or introduce technical artifacts. Due to the selection of “consistent protein spots”, the significant differences observed in the comparative analyses provide stronger evidence of a real change occurring under the treatment conditions, rather than as a result of the inherent technical variation of the system.

In this study we provide strong evidence supporting the existence of ozone related effects. Indeed, during the time course of this experiment we measured and controlled important confounding factors such as soil physical and chemical properties, morphological traits of plants and occurrence of pests (elucidated in more detail in the Supplementary table). Since the distributions of values of these confounders were statistically undistinguishable between treatment and control groups (see the Supplementary table for more details, as well as the publications [22,23,34]), we argue that the observed results are specifically associated with the ozone conditions under which the plants were grown.

Although previous proteomic studies on ozone exposure in plants have provided new insights into molecular response mechanisms, most of them have focused on short-term fumigation periods. As an extension of previous short-term ozone fumigation studies and of our snap-shot study of this experiment [35], the present work provides new insights into the modulation of proteins in beech leaves following long-term ozone exposure under natural field conditions.

Responses of juvenile beech trees after four vegetation periods under twice ambient ozone exposure at the protein level were clearly indicated. Interestingly, 43 days after the end of ozone exposure, all of the proteins except for one phosphoglycerate dehydrogenase had recovered from the effects of the ozone treatment. This result mirrored the molecular plasticity of beech trees observed following twice ambient ozone exposure. However, the possibility cannot be ruled out that protein abundances for this harvested time point decreased similarly in the control and treated samples due to seasonal factors.

In the following subsections, we discuss the proteins showing a significant difference in abundance between the ambient ozone and the twice ambient ozone fumigation treatments. For each protein, the corresponding spot number is provided in the text in brackets and is indicated in Table 1 as a reference for their specific identity. As carbon metabolism was strongly affected in the ozone-treated leaves, we provide a schematic sketch of the changes induced in this molecular process by twice ambient ozone exposure in Fig. 5.

4.1. Photosynthesis-related proteins

It is widely accepted that the photosynthesis reactions of many plant species are impaired by higher concentrations of

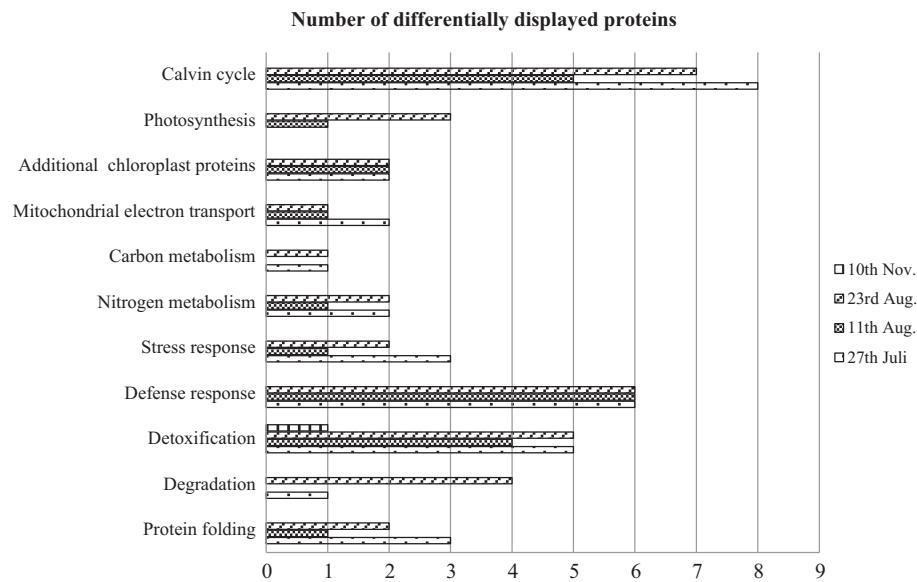


Fig. 3 – Bar diagram showing the numbers of proteins displaying differentially increased and decreased abundances identified under the mass spectrometric approach. Proteins are classified according to their biological functions for each individual time point.

the molecular response of European beech to twice ambient ozone exposure. Although the synthesis of PR proteins has been described under both biotic and abiotic stressors, the mechanisms of its regulation are not well understood. One likely cause of a common mode of modulation in response to different stressors could be evolutionary pressure toward the protection of plants against different pathogens and abiotic stresses [48].

Regarding stress-related responses, twice ambient ozone strongly influenced the synthesis of polyphenol oxidase (PPO, spot eID:0031^{27,07}, 11,08, 23,08) during July and August. This result is supported by previous studies indicating induced activity of PPO in plants under stress, wounding and pathogen

attack [49,50], thus substantiating the role of PPO in resistance to abiotic stress and pathogens.

With respect to this protein category, overall accumulation of an ankyrin repeat domain-containing protein (spot eID:0677^{27,07}) and an adenosine kinase (ADK, spot ID:0812^{27,07}, 23,08) was found in the twice ambient ozone-treated samples. Interestingly, an ankyrin repeat domain-containing protein was proposed to be regulator of JA and SA [51], which are molecules involved in the response to different stressors via containing and spreading leaf lesions and cell death [16,17,52]. The increased amounts of PPO and the possible increase in the levels of JA, and SA support the hypothesis that different

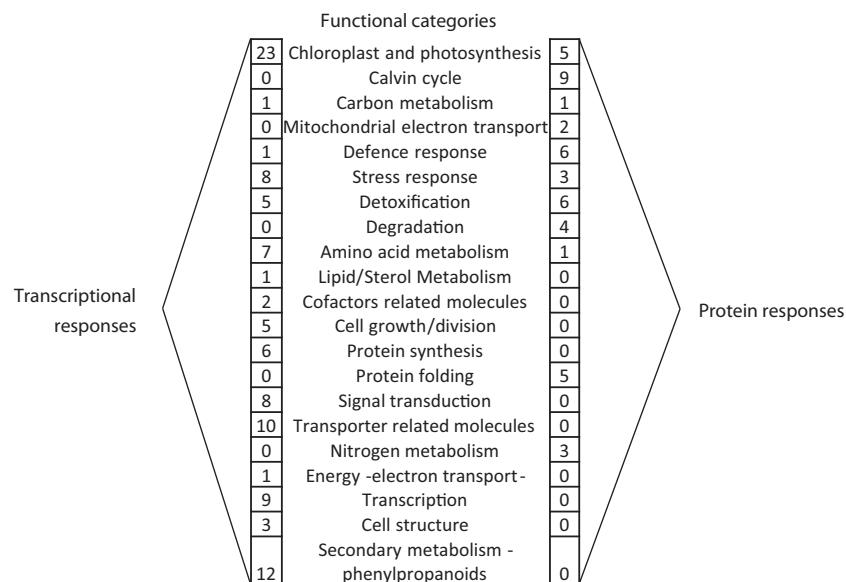


Fig. 4 – Number of transcripts (left) and proteins (right) that changed in abundance in twice ambient ozone exposed beech leaves compared to ambient ozone exposed ones. Changes are classified according functional categories.

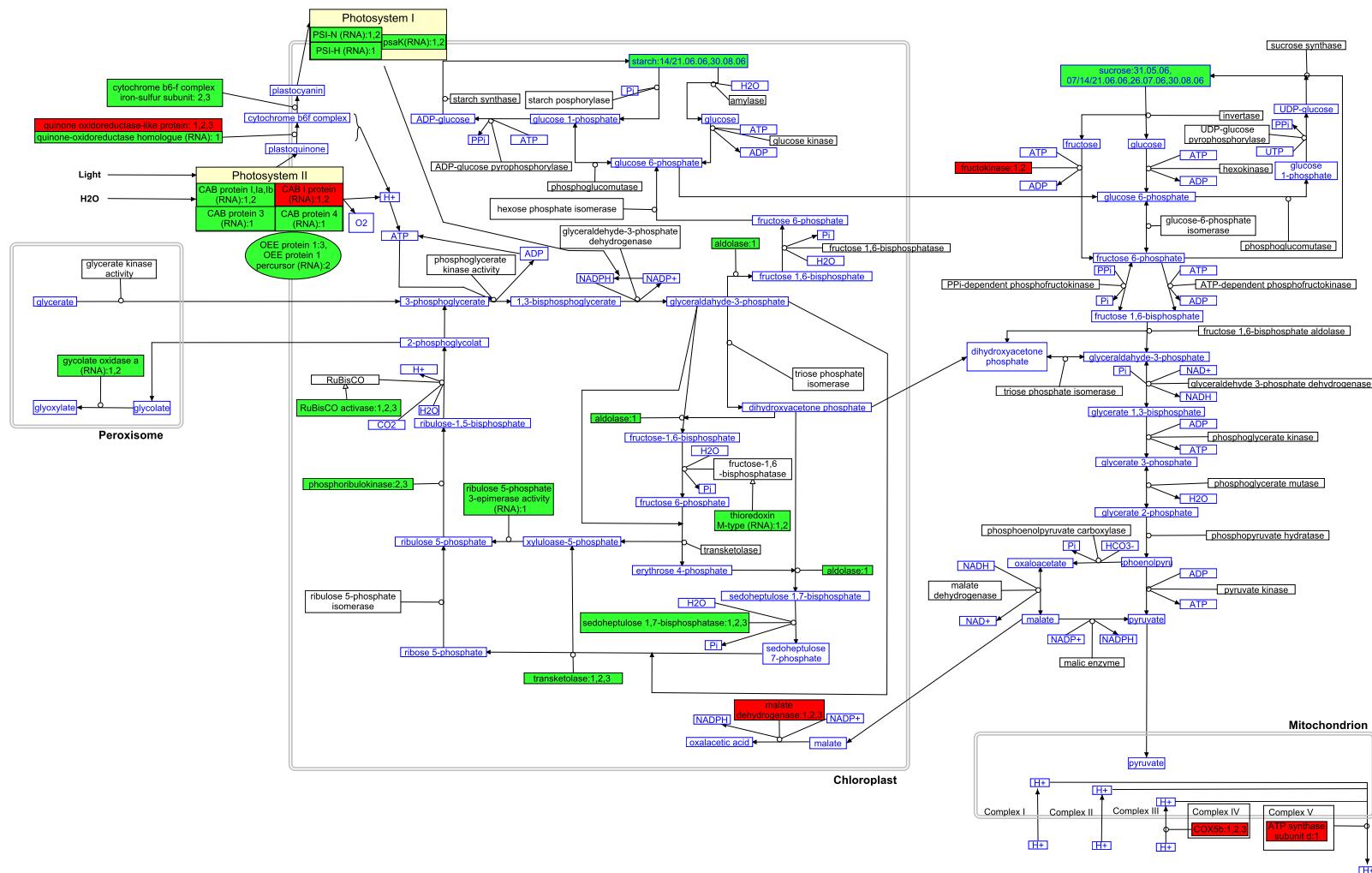


Fig. 5 – Molecular responses to twice ambient ozone exposure related to carbon metabolism in beech leaves. The initial pathway map provided by WikiPathways was manually extended using the visualization software PathVisio. Symbols are used according to the legend pane of PathVisio (catalysis reaction is indicated by a line ending in a circle, and a metabolite is depicted in blue. A white arrow indicates a stimulation). Increased and decreased abundances of molecules are shown in red and green, respectively. Changes in transcripts are indicated with the abbreviation “RNA”. The sampling dates of the modulated molecules are shown after the colon (sampling dates for transcripts and proteins are abbreviated as follow: 1: 27th of July, 2: 11th of August, 3: of 23rd August). CAB protein: chlorophyll a/b-binding protein; COX5b: cytochrome c oxidase protein subunit 5b; OEE protein: oxygen evolving enhancer protein; psaK: photosystem I reaction center subunit psaK; PSI-H: photosystem I protein subunit H (photosystem I reaction center subunit VI); PSI-N: photosystem I protein subunit N; RuBisCO: ribulose-1,5-bisphosphate carboxylase oxygenase activase.

degrading enzymes. As previously described, cysteine protease (spot eID:0124^{27.07, 23.08}) is involved in a variety of proteolytic functions and has been previously detected during the senescence processes [66,73,74]. For instance, the expression of the senescence-associated gene SAG 12, which encodes a cysteine proteinase, is specifically controlled by developmental senescence in *A. thaliana*. Its expression appears to be regulated by developmental pathways that are induced during aging, for example, when plants reduce their photosynthetic output [75]. In fact, the higher amounts of cysteine protease observed in this study correlate negatively with the decreased amounts of carbon fixation and photosynthesis-related proteins.

Moreover, dihydropyrimidinase isoenzymes (spot ID:0445^{23.08} and ID:0450^{23.08}) were up-regulated under twice ambient ozone levels. Although these isoenzymes have been associated with the cellular response to nitrogen levels, further functional characterization is needed to better understand their role in twice ambient ozone-exposed beech leaves.

4.7. Comparison of modulated transcripts and proteins

Another focus of the present study was the comparison of previously quantified levels of transcripts [19] and protein abundances in harvested leaves from the same experiment. Through this combined analysis, it was possible to better understand the molecular pathways that were affected in the beech leaves under the effects of higher ozone exposure. When the general modulated transcripts and proteins of same functional categories were compared, we observed that specific functions in cells (i.e., molecules related to chloroplasts and photosynthesis, defense responses, stress responses and detoxification) were strongly affected by the effects of long-term ozone exposure in juvenile beech trees. However, among the modulated transcripts and proteins, no direct overlap was observed. Potential explanations for this phenomenon might be as follows. Possible technical reasons include i) the selection of high-spot-resolution IEF strips in the 2-DE gels, at the expense of smaller pH ranges, thus reducing the number of analyzed protein spots. ii) In many cases, multiple proteins were identified in one spot, making comparison of modulated transcripts and proteins difficult. iii) Proteins with extremely low and high molecular weights as well as proteins with extremely basic and acidic characteristics are difficult to separate via 2D-PAGE, thus limiting the number of identified proteins. Biological reasons for this low overlap may be related to post-transcriptional regulators (i.e., miRNA interactions) causing translational repression and gene silencing and post-translational modifications that may affect the half-life of proteins [76,77].

5. Concluding remarks

The results presented here confirm to a certain degree molecular responses on other plant species, i.e., poplar, rice, and soybean, treated with short-term elevated ozone concentrations. The molecular pathways that lead to the development

of oxidative responses are rather complex. However, different plants species appear to respond in a similar way, most likely by reducing CO₂ fixation and increasing respiratory processes, as indicated in our study.

Furthermore, the presented proteomic approach was supported by earlier work focused on transcriptome analyses and non-structural carbohydrates. Most of the identified transcripts/proteins related to photosynthesis and carbon fixation showed decreased abundance, while various transcripts/proteins implicated in detoxification processes and defense and stress reactions were up-regulated. These observations suggest that energy, which was decreased following twice ambient ozone exposure, was reallocated and directed toward the repair, detoxification, and maintenance of cell structures.

Given the huge amount of decreased abundances of transcripts/proteins related to the photosynthesis and Calvin cycle and the large carbon investment into defense mechanisms, it could be expected that beech growth was affected by the enhanced ozone regime. In fact, the molecular results observed in this experiment are mirrored in the stem area increment of beech trees grown in lysimeters during the year 2005 and 2006. For this period of time the stem area increment was significantly reduced under twice ambient ozone exposure to 54–71% compared to trees grown under ambient ozone exposure [34].

As this study represents the first proteomic analysis in European beech trees exposed to enhanced ozone concentrations, the question remains to which degree protein responses differ between trees from different ontological stages. It has been reported that the large amount of transcriptional responses observed in this experiment stand contrast with the very weak transcriptional responses in mature beech trees exposed to twice ambient ozone exposure during the same experimental year [78]. This differences might be explained by the higher levels of antioxidants and photosynthetic pigments observed in mature beech trees [78].

Ultimately, the combination of several approaches including transcriptomics, differential proteomics, analysis of non-structural carbohydrates, and morphological effects contributes to a more global picture of how beech trees react under increased ozone concentrations.

Transparency document

The Transparency document associated with this article can be found, in the online version.

Acknowledgments

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