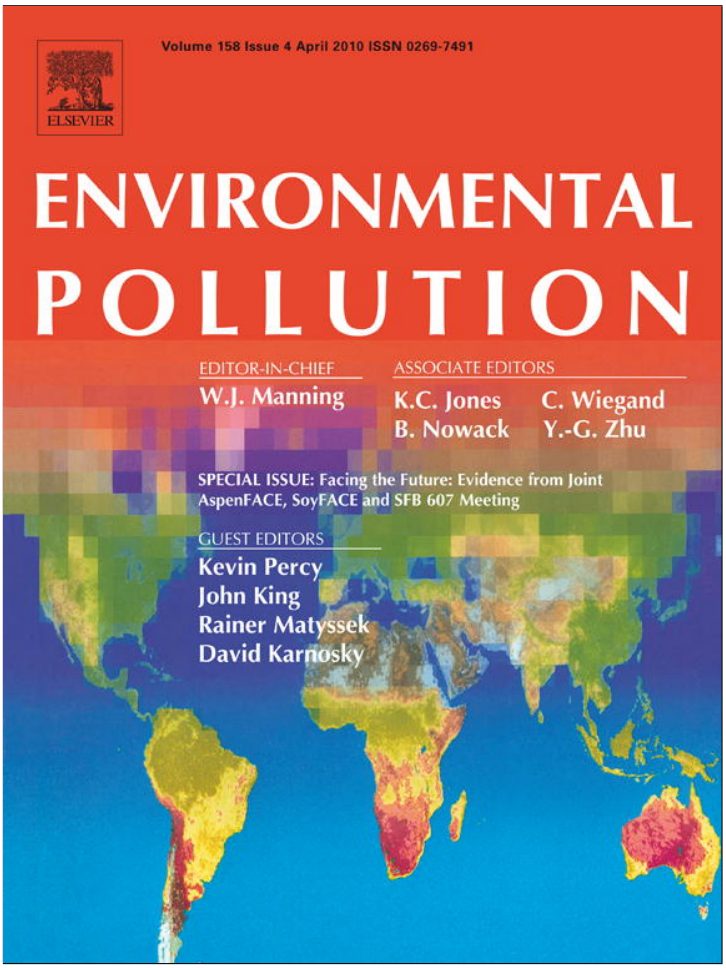

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Gene expression responses of paper birch (*Betula papyrifera*) to elevated CO_2 and O_3 during leaf maturation and senescence

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Clustering analysis of birch leaf gene expression data reveals differential responses to O_3 and CO_2.

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ABSTRACT

Gene expression responses of paper birch (*Betula papyrifera*) leaves to elevated concentrations of CO_2 and O_3 were studied with microarray analyses from three time points during the summer of 2004 at Aspen FACE. Microarray data were analyzed with clustering techniques, self-organizing maps, *K*-means clustering and Sammon's mappings, to detect similar gene expression patterns within sampling times and treatments. Most of the alterations in gene expression were caused by O_3, alone or in combination with CO_2. O_3 induced defensive reactions to oxidative stress and earlier leaf senescence, seen as decreased expression of photosynthesis- and carbon fixation-related genes, and increased expression of senescence-associated genes. The effects of elevated CO_2 reflected surplus of carbon that was directed to synthesis of secondary compounds. The combined $CO_2 + O_3$ treatment resulted in differential gene expression than with individual gas treatments or in changes similar to O_3 treatment, indicating that CO_2 cannot totally alleviate the harmful effects of O_3.

1. Introduction

Field experiments in natural environmental conditions with multiple, simultaneous stresses provide novel information about plant status in changing environmental conditions that is fundamental in understanding plant responses to global change. Long-term field experiments are essential especially for trees because of the differential responses of young saplings from mature trees (Oksanen, 2003). The rise of concentration in the greenhouse gases CO_2 ($[CO_2]$) and ozone ($[O_3]$) causes changes in the gene expression (GE) of deciduous trees (Gupta et al., 2005; Druart et al., 2006; Jehnes et al., 2007; Olbrich et al., 2005; Rizzo et al., 2007; Taylor et al., 2005; Wustman et al., 2001), which differ in acute and long-term experiments. The difference in GE caused by these greenhouse gases can be small, particularly with acclimated trees, and yet these differences are pivotal for the metabolism of the trees.

Microarrays have been used for detection of differences in GE between two conditions or time series. Traditionally, analyses have been made in consecutive phases, starting with statistical analysis to produce lists of significantly under- or over-expressed genes. The gene lists are usually restricted by using a cutoff, grouped with clustering methods to find patterns or correlations in the GE values, and finally, in the biological processes (Clarke and Zhu, 2006; Dopazo, 2006). The application of this approach to identify differentially expressed genes causes a vast loss of information because a large number of false negatives is excluded from the data to eliminate most of the false positives (Dopazo, 2006; Galbraith, 2006). In addition, the fold change-limits exclude genes with more subtle changes in expression (Curtis et al., 2005) and can lead to invalid results in the cases when the transcript levels are very low or above detection level (Clarke and Zhu, 2006).

System biology approaches require knowledge on complete biosynthesis and regulatory routes, for which the information on both the stability and the changes of GE is equally important.

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Instead of using traditional cutoff lists, multivariate and clustering methods can be used in GE analysis. These methods enable the analyses of large-scale GE data without discarding information with data filters. Multivariate methods, such as hierarchical clustering (Eisen et al., 1998), self-organizing maps (SOMs) (Kohonen, 1995), *K*-means (Hartigan, 1975) and Sammon's mapping algorithm (Sammon, 1969) have been used in analysis of GE data (Törönen et al., 1999). Lately, principal component analysis (PCA) and linear regression methods were used in order to combine aspen leaf transcriptional profiles with weather factors and to explain leaf developmental patterns (Sjödin et al., 2008).

We have studied the response of paper birch leaf GE to elevated [CO_2] and [O_3] during leaf maturation and senescence. The hypotheses were as follows. (1) Elevated [CO_2] cause accumulation of carbohydrates, defense chemicals, cell wall components and delay leaf senescence (Karnosky et al., 2003; Peltonen et al., 2005; Riikonen et al., 2004, 2008a; Taylor et al., 2008). (2) Elevated [O_3] induces oxidative stress in leaves causing decreased photosynthesis and activates defense reactions (Karnosky et al., 2003, 2005; Kontunen-Soppela et al., 2007). If the O_3 exposure is long, repair mechanisms are turned on. Because chemical defense is energetically demanding and carbon uptake is reduced, leaf senescence is activated earlier under elevated [O_3]. Alternatively, the senescence-associated processes, and remobilization and storage of carbohydrates and nutrients, may not be completed (Uddling et al., 2005). (3) In the combination of elevated [CO_2] + [O_3], the O_3-caused damages are not seen or they are smaller, due to closure of the stomata under elevated [CO_2] and thus decreased [O_3] uptake by the leaves (Karnosky et al., 2003; Riikonen et al., 2008a). On the other hand, the CO_2-stimulated "positive carbon pressure" may provide energy and defense chemicals, and enable leaves to repair the O_3-caused damages (Oksanen et al., 2007; Riikonen et al., 2004).

GE of leaves was studied with microarrays of samples collected from the long-term O_3 and CO_2 fumigation experiment Aspen FACE (Dickson et al., 2000; Karnosky et al., 2005), where acclimation to the effects of these gases has occurred. To identify distinct groups of expression patterns the obtained microarray data was clustered with self-organizing map (SOM). Since SOM clusterization produced a fixed amount of GE groups, the optimal segmentation of groups was retrieved by testing several *K*-means clustering values and ranking them with Davies–Bouldin validity index. The obtained expression profile groups were visualized with Sammon's mapping and linked to the treatments and sampling times of the experiment. Finally the groups were analyzed for enrichment of Gene ontology terms to reveal the biological function of the GE changes.

2. Materials and methods

2.1. Plant material

Paper birch (*Betula papyrifera*) leaves were collected from long-term O_3 and CO_2 fumigation experiment Aspen FACE established near Rhinelander, Wisconsin WI (W89.7°, N45.7°) (http://aspenface.mtu.edu/) (Dickson et al., 2000; Karnosky et al., 2005). The site contains 12 FACE rings (30 m diameter) set up as a 2 by 2 factorial design with three rings receiving supplemental CO_2, three rings receiving supplemental O_3, three rings receiving both supplemental CO_2 and O_3, and three rings receiving ambient air (control rings). Birches originating from seeds collected in Michigan (Dickson et al., 2000) have been exposed to elevated [CO_2] (target 550 ppm) and O_3 (target 1.5× ambient) singly and in combination during the growing season since 1998. During year 2004 the average [O_3] in the O_3-exposure rings were 1.2× above ambient (seasonal average [O_3] 40.2–41.7 ppb, depending on the exposure ring), the daytime [CO_2] average of all CO_2-exposure rings was 512 ppm (seasonal average daytime [CO_2] 501.2–518.0 ppm, depending on the exposure ring) and the exposure lasted for 150 days. The summaries of O_3 exposure for each ring at each exposure month are available at http://aspenface.mtu.edu/FACE O3 Summaries 1998-2007.pdf and for CO_2 at http://aspenface.mtu.edu/FACE CO2 Summaries 1998-2007.pdf. Further information about the field site, experimental design and technical details can be found in Dickson et al. (2000). Leaf samples consisting of 5–7 fully mature short shoot leaves per tree of paper birch have been collected from three trees in each ring in mid July, August and September 2004.

2.2. RNA-extraction

RNA was isolated from birch leaves according to Chang et al. (1993) with the following exceptions: Frozen leaves (200–300 mg) were homogenated first in liquid N with sand and then in prewarmed extraction buffer where 1% Tween® 80 (Fluka) was added. The homogenates were incubated at 65 °C for 50 min–2 h, shaken for 15 min, and incubated at 65 °C for 15 min before the extraction was continued with chloroform: IAA as in Chang et al. (1993). An additional purification was made for the ethanol-precipitated RNA. RNA was dissolved in 100 μl nuclease-free water, 10 μl 20% PVP was added, mixed briefly and the mixture was centrifuged 10,000*g* at room temperature for 5 min. The supernatant was then purified with aRNA spin columns (Ambion, product no. 10051 G) according to the manufacturer's instructions in AminoAllyl MessageAmp aRNA Kit (Ambion). The RNA samples from the three different trees in each ring were pooled. The pooled RNA sample (1 μg) was amplified with AminoAllyl MessageAmp aRNA Kit according to the manufacturer's instructions.

2.3. Hybridization on microarrays

The hybridization design of the experiment is shown in Fig. 1. The RNA was extracted separately for each tree sampled, but pooled RNA of three trees in each exposure ring was used for microarray analyses, and the rings were used as replicates for the analyses. All hybridizations were done with dye-swaps.

cDNA-microarrays used in the study consisted of 8153 *Populus euphratica* ESTs representing approximately 6340 distinct genes, originating from leaf, shoot and root control libraries and several cDNA libraries representing genes induced in response to, for example, elevated CO_2 and O_3, salt, cold, flooding, drought and other abiotic stresses (Brosche et al., 2005). The cDNA was spotted on three replicates on epoxy–silane coated Nexterion® HiSens E–borosilicate glass with reflective coating (Schott Ag, Mainz, Germany), the size of 75.6 mm × 25.0 mm, at Turku Finnish microarray center. The array design is available in ArrayExpress (http://www.ebi.ac.uk/microarray-as/aer/entry) with the accession number A-MEXP-1043.

The aRNA was labeled with Cy3 or Cy5 (Amersham Biosciences) before the hybridization according to the instructions in the AminoAllyl MessageAmp aRNA Kit (Ambion). The slides were prehybridized in prehybridization buffer (2% BSA, 5* SSC, 0.1% SDS) for 30 min–3 h at 65 °C. The arrays were hybridized in 50% formamide, 5*SSC, 0.1% SDS, 5*Denhardt's solution (Sigma) and 10% Herring sperm (1 mg/ml) (Sigma) for 16–18 h at 42 °C. The slides were then washed and scanned

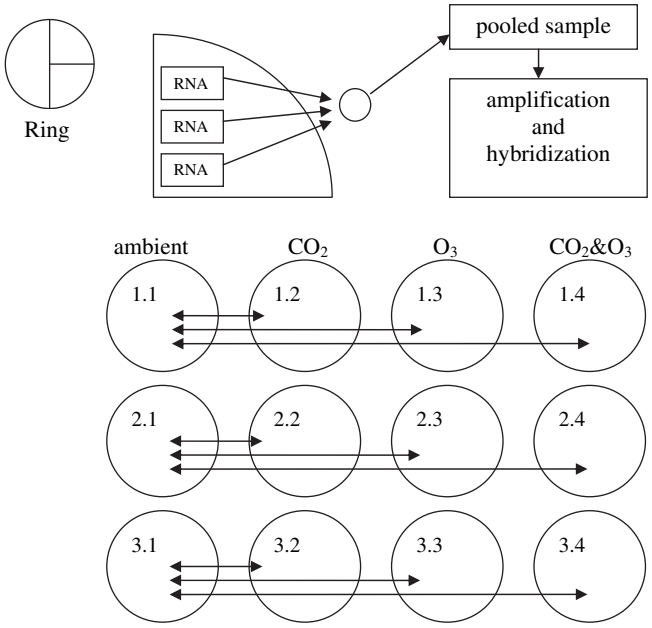


Fig. 1. Hybridization design of the experiment. Samples were collected in 3 replicate birch trees from each ring, and RNA samples were pooled for amplification and hybridization. Three sets of treatments: elevated CO_2, elevated O_3, and combined elevated $CO_2 + O_3$ were hybridized with the ambient control within each set ($n = 3$).

immediately after the hybridization with scanner (ScanArray 5000, Perkin–Elmer, USA) at 635 nm and 532 nm.

2.4. Microarray data analysis

The scanned images were analyzed in GenePixPro 5.0 (Axon Instruments, Union City, CA, USA). Median of spot fore-and background intensities was used. Background correction was done with Normexp algorithm (R, LIMMA package) (Smyth, 2005) with the offset of 50 to prevent low intensity dampening.

Median averaging was used for the triplicate spots on each slide, and the data were normalized with log2-transformation followed by LOWESS normalization (Quackenbush, 2002), median averaging the dye-swap pairs (Yang and Speed, 2002) and averaging the sample replicates from each treatment ring. A total of 7635 ESTs data on each slide were put forward to the data analysis. Every preprocessing step was done similarly to all the data and verified by data visualization (boxplots, Sammon's mappings) to gain control over the preprocessing and normalization methods.

To identify distinct groups of expression patterns the data were clustered with self-organizing map (SOM), using Visual Data software (Visipoint Ltd, Finland). SOM clusterization produced a fixed amount of GE groups (256). The partitioning of the groups was studied with *K*-means algorithm (Hartigan, 1975). Optimal grouping was retrieved by testing *K*-values from 2 to 70 and ranking them with Davies–Bouldin validity index (DB-index) (Davies and Bouldin, 1979). The *K*-value with the lowest DB-index was chosen for grouping the SOM-clusters. The expression profile groups for the archetypal SOM vectors were drawn, and visualized with Sammon's mapping algorithm that preserves the interspatial pattern (Sammon, 1969). The SOM neurons of each cluster are visualized with Sammon's mapping (Supplemental Fig. 1). The data for GE patterns of the ESTs in different treatments and sampling times, sorted in 27 clusters, are presented in Supplemental Data Table 1.

2.5. Functional analysis of GE in clusters

The obtained *K*-means clusters were classified according to the GE pattern within each cluster (Supplemental Fig. 2). The criteria for change from the ambient control were set as follows: The centrum of the cluster has to deviate from 0 (no GE change from ambient control) by at least 0.15 and >95% of the ESTs within the cluster must deviate from 0 to similar direction, i.e. having either decreased or increased GE.

Gene ontology (GO) term annotation and function-based analysis of gene groups from clustering were performed using the software Blast2GO (Conesa et al., 2005; Götz et al., 2008). GO terms for each of the three main categories (biological process, molecular function, and cellular component) were obtained from sequence similarity (*E*-value $1e^{-5}$) of the EST sequences on the array. The application default parameters were used for the other analyses. The combined graph functions were used to find GO terms for "Biological processes" in the *K*-means clusters data. This method is suggested for analyses of collective biological meaning for a set of sequences (Conesa and Götz, 2008). The node information score was set as 6 and used as a cutoff for the GO classes reported in Table 1.

2.6. RT-PCR

The microarray results were verified with quantitative real time RT-PCR. Similarly to microarray analyses, pooled RNA of three trees in each exposure ring was used for the reaction and the rings were used as replicates. The RNA was first treated with TURBO DNA-free DNase (Ambion) according to manufacturer's instructions. Reverse transcription was performed with 1.5 μg of total RNA with 1:1 mixture of SuperScript II and SuperScript III (Invitrogen) at 46 °C overnight. The 10 μl RT-reaction was diluted to 90 μl and 1 μl was used as a template for the PCR using SYBR green I mastermix (Roche). Quantitative PCR was performed with LightCycler® 480 System (Roche). The primer pairs used for the analyses were designed using silver birch EST-library (Aalto and Palva, 2006) and available sequences of *Betula*-species in GenBank. The primer sequences used are carbonic anhydrase left 5′-TGGATTTC-CAACCAGGAGAG, right 5′- TCTGATCAAAGGGTGGAACC; Rubisco Small subunit left 5′-GCAATTGGCCAAGGAAGTAG, right 5′- CTCCAACTCGAATTCCAAGC; metallothionein left 5′-GGGTTGCACCAGTGAAGATT, right 5′- CAGCTGCAGTTTGATCCACA; catalase left 5′-CGTCTCGGACCAAACTACCT, right 5′- TTGTTGTGATGAGCGCATTT; alanine-glyoxylate aminotransferase left 5′-GGGTTTTCTTTGACTGGAATGA, right 5′-AAGGGGTGTATGGCCAAAAT; glucosidase II α-subunit (Brosche et al., 2005) left 5′-TAAGGGAGGCATTACCCACA, right 5′- CCCTTTGGAAGGCAGGAATA; actin left 5′-TGGTCAAGGCTGGGTTTGC, right 5′- CTGACCCATCCCAACCATGA. The raw threshold cycle (Ct) values were normalized against actin and glucosidase II alpha. The reference genes were selected on the basis that they were not influenced by the treatments according to the array data, and that their Ct values were similar in samples of treatment and control rings. The obtained normalized ΔCt values were used to calculate the ΔΔCt, i.e. the difference between expression levels between treatments and ambient within each time point (Livak and Schmittgen, 2001). The data are presented in Table 2.

3. Results

The microarray data were analyzed to identify distinct groups of expression patterns within each treatment and time of sampling. Rather than acute responses, the changes in GE show a steady-state situation, where acclimation to the effects of the gases has occurred, and the changes in GE were expected to be minor in magnitude. Therefore, all the original information of the microarray data was maintained during the data analyses process, including all possible biological and technical replicates and data of all the ESTs in the array. However, tree to tree variation in response to treatments cannot be shown, because the samples were pooled within each exposure ring and the three rings were used as replicates. The normalized data were clustered with self-organizing map (SOM) to a predetermined amount of GE groups (256 neurons). The SOM neurons of each cluster are visualized with Sammon's mapping (Fig. 2) that presents a two-dimensional figure of the whole data. The optimal segmentation of these SOM neurons with *K*-means clustering produced 27 clusters, each of which contains the expression data of 78–547 ESTs (Table 1). The Sammon's mapping, in combination with the GE patterns of individual clusters, was used to find the clusters that show GE changes in a treatment at a certain time (Fig. 2, Table 1), and the 27 clusters were analyzed for over-represented GO classes for biological processes (Table 1).

The clusters that did not show any relation to a treatment at a time (clusters 2, 3, 6, 9, 10, 19, 24), included genes that could be regarded as "household genes", and in the GO-classification many of the clusters were related to primary metabolism (Table 1).

The correlation of quantitative RT-PCR results with the microarray results was generally high, although the absolute fold change differed between the two methods for genes that had high expression levels, e.g. Rubisco Small subunit (Table 2).

3.1. Effects of treatments

3.1.1. Elevated [O_3]

Based on the Sammon's mapping, the most distant GE profiles were produced by elevated [O_3] exposure (Fig. 2). The number of clusters that had significant change in GE was greatest with elevated [O_3], either with or without elevated [CO_2] (Table 1). The magnitude of change in GE was the greatest in the O_3-exposed samples, ranging from 3.5-fold increase (log2 fold change (FC) 1.8, EST for ferritin in cluster 25) to 0.29-fold decrease (log2 FC -1.8, carbonic anhydrase in cluster 22).

O_3-induced GE changes in July (clusters 4 and 7) were related to increased transport and to photorespiration (Table 1). In cluster 4, expression of cell growth and cell expansion-related genes, such as actin-related proteins, pectin acetylesterase and ascorbate oxidase increased. Ascorbate oxidase can also be linked to ROS formation, as well as arginine decarboxylase, metallothioneins and ACC synthase that grouped in cluster 4. Photorespiration-pathway (catalase, aminotransferase, glycolate oxidase, hydroxypyruvate reductase, GAPH) (cluster 7) and electron transport-related (alternative oxidase AOX, proton gradient regulation) (cluster 4) GE increased in O_3 exposure in July. Expression decreased in elevated [O_3] in July in photosynthesis light harvesting genes (cluster 1), transcription and translation-related genes (clusters 1 and 14) as well as proteolysis genes indicating both decreased protein synthesis and turnover (Table 1). Several alpha- and beta-tubulin ESTs were decreased in expression, which may be a sign of reduced cell division.

In the August sampling, elevated [O_3] induced GE for carbohydrate metabolism. Especially genes associated with glycolysis and phenylpropanoid metabolism, lignin (cinnamoyl-reductase, peroxidase) and cellulose biosynthesis (reversibly glycosylated polypeptide) genes were increased in expression (cluster 17).

Table 1
K-means clustering results. The cluster identification number (ID), number of ESTs in each cluster, sampling time of the change in gene expression (GE) (time), treatment showing increased or decreased GE, gene ontology (GO)-classes of biological processes classification that show significant over-representation (at node score >6) and biological significance of the GE changes within each cluster. An asterisk in column Fig. 2 refers to Fig. 2 that shows the cluster in a Sammon's mapping and its GE profile.

ID	ESTs	time	increase	decrease	Fig.2	Over-represented GO-classes (Biological processes)	Biological significance
4	220	Jul	O_3			cellular protein metabolic process; transport; electron transport	ROS-related defense reactions to O_3, cell growth and expansion
7	114	Jul	O_3		*	transport; photorespiration	increased photorespiration
		Jul	CO_2+O_3				
		Sep		O_3			decreased photorespiration
1	188	Jul		O_3	*	translation; electron transport; photosynthesis (light harvesting); response to stress	downregulation of photosynthesis light harvesting and electron transport
		Jul		CO_2+O_3			
		Jul		CO_2			
		Aug		O_3			
		Aug		CO_2+O_3			
14	330	Jul		O_3	*	regulation of transcription; transcription; response to stress; translation ; proteolysis; transport; microtubule-based movement	cell division (microtubule)
		Jul		CO_2+O_3		protein polymerization	
17	246	Aug	O_3			cellular protein metabolic process; carbohydrate metabolic process; protein folding	lignin and cell wall synthesis, photorepair
27	147	Aug	O_3			transport; cellular protein metabolic process	
21	78	Aug		O_3	*	translation; photosynthesis; transport	downregulation of photosynthesis
		Aug		CO_2+O_3			
		Sep		CO_2+O_3			
25	162	Sep	O_3		*	proteolysis; protein modification; response to stress; transport	catabolism of proteins, senescence
22	121	Aug		O_3	*	photosynthesis(light reaction); translation; reductive pentose-phosphate cycle; glycolysis	downregulation of photosynthesis and carbon assimilation
		Sep		O_3			
		Sep		CO_2+O_3			
15	270	Sep		O_3		translation; ribosome biogenesis and assembly; regulation of transcription (DNA dependent); photosynthesis; response to hormone stimulus	downregulation of photosynthesis, translation and cell response, senescence
11	180	Jul	CO_2+O_3			primary metabolic process; transport	primary metabolism
16	79	Jul	CO_2+O_3			-	many unknown genes
20	538	Jul	CO_2+O_3			carbohydrate metabolic processes; regulation of transcription; protein amino acid-phosphorylation; response to stress; post-translational protein modification; glycolysis	synthesis of steroids and phenylpropanoids
8	323	Jul		CO_2+O_3		cellular protein metabolic process; translation; regulation of transcription; protein amino acid-phosphorylation; proteolysis; carbohydrate metabolic process; response to stress	
5	500	Aug		CO_2+O_3		translation; transport; regulation of transcription (DNA-dependent); transcription; protein amino acid-phosphorylation; protein folding; proteolysis; response to hormone stimulus	decreased transcriptional and translational activity; decreased protein modification
26	276	Sep		CO_2+O_3		regulation of transcription; protein a-a-phosphorylation; response to stress; electron transport	transcription factors
18	277	Jul	CO_2			regulation of transcription; transport; response to stress; protein amino acid-phosphorylation	increased N-fixation
23	567	Aug	CO_2			transport; translation; protein amino acid-phosphorylation; post-translational protein modification; regulation of transcription (DNA-dependent); proteolysis; RNA processing; response to auxin stimulation	increased glycolysis and TCA-cycle, phenylpropanoid synthesis
12	320	Aug		CO_2		translation; transport; regulation of transcription (DNA-dependent); regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process; ribosome biogenesis and assembly	decreased oxidative phosphorylation, decreased translational activity
13	350	Sep		CO_2		transport; translation; regulation of transcription (DNA-dependent); response to stress	decreased signaling and regulation
2	547	-				translation; proteolysis; protein amino acid-phosphorylation; response to stress	cellular protein metabolic process
3	349	-				response to stress; cellular protein metabolic process; proteolysis; protein transport	macromolecule metabolic processes, transport
6	271	-				carbohydrate metabolic process; translation; regulation of transcription (DNA-dependent)	cellular metabolic processes
9	296	-				cellular protein metabolic process; translation; response to stress	primary metabolic processes
10	318					protein amino acid-phosphorylation; post-translational protein modification; regulation of transcription; protein transport;	primary metabolic processes
19	313	-				transport; translation; proteolysis	cellular metabolic processes
24	255	-				transport; response to stress	metabolic process

Table 2
Comparison of microarray (array) and real time RT-PCR (PCR) data for selected genes. The values are shown as log2 change against the ambient control.

Gene	CO_2						O_3						$CO_2 + O_3$					
	July		August		September		July		August		September		July		August		September	
	Array	PCR	Array	PCR	Array	PCR	Array	PCR	Array	PCR	Array	PCR	Array	PCR	Array	PCR	Array	PCR
Carbonic anhydrase	0.02	−0.19	−0.30	−0.60	−0.41	−0.36	−0.04	0.57	−0.45	−1.53	−1.53	−2.72	0.25	0.66	0.04	0.06	−0.35	−0.35
Rubisco Small subunit	0.03	−0.27	−0.09	−0.37	−0.13	−0.06	0.04	0.63	−0.16	−0.96	−1.02	−1.58	0.06	0.68	0.06	0.00	−0.17	−0.07
Metallothionein	0.82	0.59	0.32	0.98	0.33	2.02	0.32	1.63	−0.03	0.59	0.01	1.76	0.21	0.21	0.20	1.24	−0.17	0.51
Catalase	−0.16	−0.45	−0.14	−0.43	−0.15	0.09	0.58	1.91	−0.21	−1.00	−0.41	−0.56	0.60	2.13	−0.06	−0.30	−0.16	0.52
Alanine-glyoxylate aminotransferase	0.13	−0.23	0.21	−0.31	0.03	0.20	0.10	1.24	0.13	−0.79	−0.70	−1.27	0.40	1.58	0.14	0.13	−0.34	0.13

Contrastingly, GE of carbon fixation and/or glycolysis enzymes, such as Rubisco, Rubisco activase, and chloroplast glyceraldehyde-3-P-dehydrogenase decreased (clusters 21 and 22) (Table 2). The expression of different chloroplast and photosystem-repair-related proteins (6-4 photolyase, DeGP2 protease) and chaperonins increased (cluster 17) simultaneously when the expression of photosynthesis-related genes (PSI and PSII) was strongly decreased (clusters 21 and 22).

In September, elevated [O_3] induced different Cys and Asp proteases, and expression of other genes related to proteolysis, including ubiquitin (cluster 25). The large amount of ubiquitin related ESTs in cluster 25 caused over-representation of the GO-class "response to salicylic acid stimulus" in response to elevated [O_3] in September. GE of transport-related proteins, such as vacuolar sorting receptors, and various senescence-associated genes increased. TCA-cycle enzyme genes, citrate synthase, succinate dehydrogenase and PEP carboxykinase, as well as glycolysis-related aldehyde dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase and alcohol dehydrogenase increased in expression in September samples (cluster 25). Elevated [O_3] decreased expression of the same genes in August and September since cluster 22 was classified to both sampling times (Table 1). The magnitude of change was even greater in September, the center of the cluster changed from −0.38 in August to −0.69 in September (Fig. 2b). Interestingly, this is the same set of genes that increased in expression in July (cluster 22). Translation-related and ribosomal protein GE, and expression of many transcription factors decreased in September (cluster 15).

3.1.2. Elevated [CO_2]

The effects of elevated [CO_2] on leaf GE were smaller in magnitude than with O_3, ranging in July from 2.5- to 0.5-FC (1.3 to −1.0 log2 FC), and the changes were greater in July and August samples than in September (Table 1). Except for cluster 21 that showed a universal decrease in GE in most of the samplings, the elevated [CO_2] showed specific changes in GE.

CO_2-induced changes in July were seen as increased expression of nitrogen fixation genes (nitrate reductase, glutamate synthase, glutamine synthase) and metallothioneins (Table 2) (cluster 18). In August expressions both TCA-cycle and glycolysis-route genes were increased (cluster 23), but oxidative phosphorylation GE was decreased (cluster 12). Genes responding to auxin stimulus and phenylpropanoid biosynthesis genes (cluster 23) were increased in expression in August at elevated [CO_2]. Based on the clustering analysis, the smallest amount of changes in GE pattern was observed in September at elevated [CO_2]. These changes include decrease in some photosynthesis and oxidative phosphorylation-related genes.

3.1.3. Combined elevated [$CO_2 + O_3$]

In combined elevated [$CO_2 + O_3$] the changes in GE resembled the O_3 treatment rather than the elevated [CO_2]-treatment, as shown by clusters 1, 7, 14, 21 and 22 (Table 1, Fig. 2). In addition to many similarities in GE to the sole elevated [O_3]-treatment, the combined $CO_2 + O_3$ treatment resulted in some specific GE changes (clusters 5, 8, 11, 16, 20, and 26) (Table 1).

In July, some carbon fixation-related genes were increased in expression (Rubisco, fructose-bisphosphate aldolase, ribulose-phosphate 3-epimerase). A great amount of GE related to secondary metabolism that was special for the combined treatment was activated in clusters 11, 16 and 20. Steroid (isopentenyl diphosphate isomerase 2, sterol methyltransferase 1,1-deoxy-D-xylulose 5-phosphate reductoisomerase, farnesyl diphosphate synthase) and phenylpropanoid synthesis (4-coumarate:ligase, peroxidase, trans-cinnamate 4-hydroxylase, cinnamyl alcohol dehydrogenase) genes were increased in expression (cluster 20). Decreased expression was mostly similar to the O_3 treatment (clusters 1 and 14), but cluster 8 included a set of genes with small changes in GE in response to combined elevated [CO_2] + [O_3].

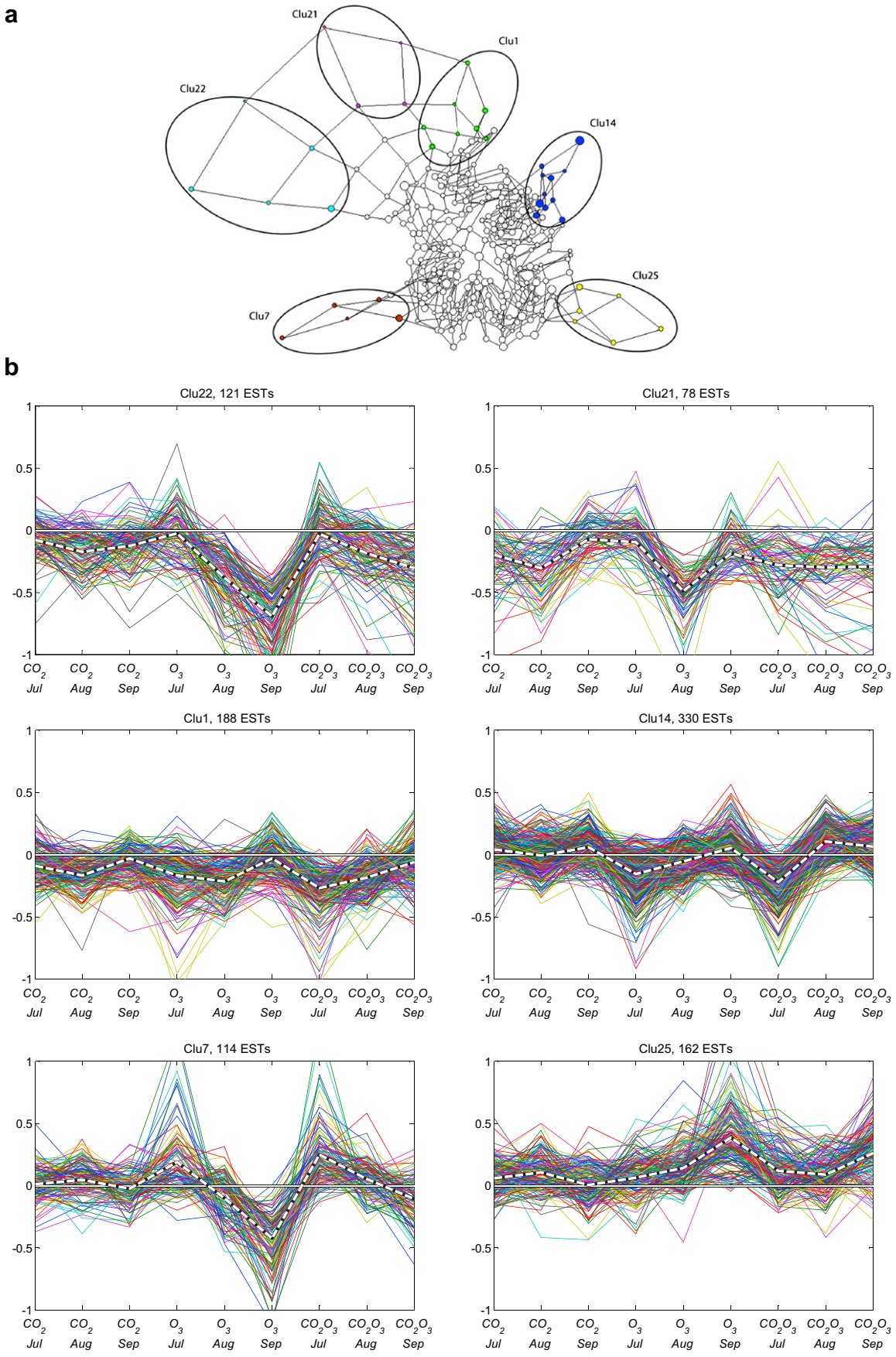
In August, the combined elevated [CO_2] + [O_3] treatment resembled the control ambient treatment, and only some changes with decreased GE were found (clusters 1, 21 similarly to O_3, and cluster 5). GE of some steroid synthesis genes (e.g. squalene epoxidase, 1-deoxy-D-xylulose 5-phosphate reductoisomerase) as well as genes of jasmonic acid biosynthesis route (lipoxygenase) decreased (cluster 5). GE of many transcription factors and hormonally regulated genes (*AIN1*, *WRKY40*, brassinosteroid receptor, etc.) decreased. The expression patterns of the combined treatment in September resembled that of both combined treatment and elevated [O_3] in August (clusters 21 and 22). Expression of many transcription factors was decreased (cluster 26) in September.

4. Discussion

Multivariate methods on GE analysis provided clusters with similar GE patterns within sampling times and treatments. Although the absolute changes in GE were rather small in the experiment where acclimation to elevated [CO_2] and [O_3] had occurred, the functional analysis of clusters proved that the clustering method was effective. By combining multivariate methods we were able to show changes in GE that could be masked by traditional methods with data filters, such as gene lists based on cutoff values.

4.1. Effects of elevated [O_3]

Exposure to O_3 is well known to cause stomatal closure, which is followed by reduced CO_2 uptake into the leaves (Wittig et al., 2007). In O_3-stressed birch leaves decreased stomatal conductance was accompanied by diminished net photosynthesis (Riikonen et al., 2008a). In the present study, impaired photosynthesis was seen as downregulation of PSI and II light harvesting complex genes in July. Downregulation of light harvesting system is an efficient and dynamic feedback mechanism to regulate and balance the overall



photosynthesis process and to provide protection against photoinhibition, when energy gain through light interception exceeds the energy use by subsequent photosynthetic reactions. Plants use a diverse set of protective systems, such as photorespiration and non-photochemical quenching (NPQ), to maintain photosynthetic balance and continuous energy flow (Niyogi, 2000). Although photorespiration may protect the photosynthetic apparatus by dissipating excess electron flow through the photosynthetic apparatus, it is producing toxic H_2O_2 (Niyogi, 2000). In July samples, elevated [O_3] increased expression of several photorespiration and NPQ-related genes indicating active protection of photosynthetic machinery. Downregulation of photosynthesis-related genes and increase in photorespiration-related GE in *Populus* leaves were also observed by Gupta et al. (2005) under O_3 treatment.

Active defense against increased oxidative stress was evident in July, when GE related to reactive oxygen species (ROS) formation, such as catalase (Olbrich et al., 2005), alternative oxidase (Pasqualini et al., 2007), and peroxidases increased. Other genes, such as ascorbate oxidase and pectin acetylesterase, that increased in GE in July can be linked to cell growth and expansion (Kato and Esaka, 2000; Sanmartin et al., 2003), while expression of cell division-associated genes (tubulin, actin-related protein) decreased. This complex action of growth-related genes can be linked to reduced size of leaves (Riikonen et al., 2008a), and epidermal cells (Riikonen et al., 2008b) in elevated [O_3]-exposed birch trees.

In August, there was a decrease in photosynthesis GE (relating both to the light harvesting as well as carbon assimilation pathways) and the photoprotection-related GE. Interestingly, many genes associated to photosynthesis, particularly photorespiration-related genes that strongly increased their expression in July, were decreased in August. In contrast, GE related to repair of damage to photosystems (DegP2 protease, Haussühl et al., 2001) and DNA (6-4 photolyase, Nakajima et al., 1998), as well as protein folding was increased.

Although many C-fixation and/or glycolysis genes were down-regulated in August, there was an increase in the expression of lignin and cell wall biosynthesis related genes. Lignin gives mechanical strength and assists in solute transportation by decreasing the permeability of walls, and impacts resistance to attack of microorganisms (Higuchi, 1997). Increased GE of lignin synthesis enzymes during O_3 exposure was recently reported in tobacco (Dauwe et al., 2007) and beech (Jehnes et al., 2007). Increased lignin content of leaves was detected by Cabané et al. (2004) in poplar and Jehnes et al. (2007) in beech, and stem wood in birch (Kaakinen et al., 2004), although lignin content of birch leaf litter did not alter significantly (Liu et al., 2005). These findings indicate that reduced carbon flux upon O_3 stress was targeted to damage repair and defense against ROS by increased lignin synthesis.

In September, the O_3 decrease in photosynthesis-related GE was even more pronounced than in August samples and was accompanied by enhanced leaf senescence and catabolism of proteins. O_3 induced earlier leaf senescence has been widely reported (Karnosky et al., 1996; Riikonen et al., 2004; Pritsch et al., 2008). Several senescence-associated genes were induced, as earlier reported in O_3-exposed *Populus* leaves at AspenFACE (Gupta et al., 2005), such as chloroplast stay-green related to chlorophyll degradation (Ren et al., 2007). In senescing leaves, glycolysis and TCA-cycle genes were activated possibly to produce energy for the degradation and transport of proteins, seen as increased expression of proteolytic enzymes (cysteine proteinase, Bhalerao et al., 2003; Gupta et al., 2005; Sillanpää et al., 2005), and ubiquitin (Yoshida, 2003).

4.2. Effects of elevated [CO_2]

The rise of [CO_2] increases net photosynthesis (Nowak et al., 2004), as observed in birches of this experiment (Riikonen et al., 2008a). In contrast to Gupta et al. (2005), who reported of an increase in the amount of transcripts of the photosynthesis light harvesting machinery in *Populus*, the GE for photosynthesis light reactions was decreased slightly in July samples of the current study. This discrepancy may be due to difference in species, time of sampling (July versus August in Gupta et al., 2005) and the weather conditions prior to the sampling.

Availability of nitrogen has an important role in the regulation of photosynthesis at elevated [CO_2]; when nitrogen becomes limiting, the rise in photosynthesis is smaller (Drake et al., 1997). Our data, however, show an increase in the GE related to nitrogen metabolism in July samples, possibly indicating increased nitrogen acquisition and N uptake from soil, previously observed in birches of Aspen FACE (Zak et al., 2007a,b). On the other hand, Zak et al. (2007a) observed no effect of CO_2 on soil organic matter. The increased GE of N uptake could be interpreted as a signal of growing N-demand to balance the C/N ratio under elevated [CO_2]. This is supported by recent results of Li et al. (2008) with *Arabidopsis*, showing correlation in the expression profiles of N-deficiency and elevated [CO_2] and imbalance of C:N metabolism.

Elevated [CO_2] enhances cell expansion-related genes, such as xyloglycan endotransglycolases (Druart et al., 2006; Gupta et al., 2005), observed here in July. This could be a sign of increased cell expansion in elevated [CO_2] that has been previously reported (Taylor et al., 2003). However, no significant change in leaf size was observed in these trees (Riikonen et al., 2008a).

In August, GE related to the TCA-cycle and glycolysis was increased in elevated [CO_2], but GE related to oxidative phosphorylation decreased, indicating a growing demand for production of carbon skeletons for biosynthetic reactions, such as amino acid, fatty acid or polyphenol synthesis. In *Arabidopsis*, both accumulation of glycolysis and TCA-cycle products and increase in the GE of enzymes in these routes were seen under elevated [CO_2] (Li et al., 2008). The increased biosynthesis of phenylpropanoid compounds, such as phenolic acids, has been reported in birch leaves in elevated [CO_2] (Oksanen et al., 2005; Peltonen et al., 2005). This is in line with our data showing increased GE of the phenylpropanoid pathway, but in contrast to data of *Populus* (Gupta et al., 2005) and soybean (Casteel et al., 2008) in FACE experiments. Particularly, myricetin glycosides and condensed tannins (i.e. protoanthocyanidins) tend to increase in birch leaves grown under elevated [CO_2] (Peltonen et al., 2005), which may be related to better carbohydrate resources and delayed senescence. Other major changes due to elevated [CO_2] were found as an upregulation of some auxin-related genes previously reported by Gupta et al. (2005) and Taylor et al. (2005), as well as increase of genes related to signaling and regulation as reported by Ainsworth et al. (2006) and Li et al. (2006, 2008) in herbaceous plants.

Fig. 2. (a) Sammon's mapping showing clustering of the 256 neurons obtained by self-organizing map (SOM) analysis and (b) SOM mappings of selected clusters. In Sammon's mapping spatial distance correlates with difference in average gene expression (GE) profile and the node circle size with the number of genes (ESTs) present in each neuron. The clusters showing GE change in a treatment at a time are shown in different colors and the numbers of clusters refer to Table 1. The encircled clusters are provided with inserted SOM mappings showing GE changes in each treatment at a time of sampling.

In September, only decreased GE was found in response to elevated [CO_2] treatment, and many of the genes were related to signaling and transcription regulation. In contrast to our previous findings with *Betula pendula* no alterations in senescence-related GE were observed (Kontunen-Soppela et al., unpublished results) although phenological data show delayed leaf senescence of trees in elevated [CO_2] (Riikonen et al., 2004; Taylor et al., 2008).

In general, the effects of elevated [CO_2] on GE of leaves were smaller than in O_3 and were emphasized in younger leaves and samples taken during July–August, corresponding to the results on *Populus* (Taylor et al., 2005).

4.3. Effects of combined elevated [CO_2]+[O_3] treatment

The combined elevated [CO_2] + [O_3] treatment caused changes that were more similar to elevated [O_3] than to [CO_2], indicating that elevated [CO_2] is not able to totally alleviate the harmful effects of [O_3] (Karnosky et al., 2003; Riikonen et al., 2005; Oksanen et al., 2005). The expression changes in combined elevated [CO_2] + [O_3] were, however, smaller compared to the O_3-induced changes. The results are in accordance to previous GE data with *Populus* in FACE experiment where the effects of combined treatment showed more resemblance to elevated [O_3] than to [CO_2] (Gupta et al., 2005), but different from our recent data from *B. pendula* in open top chambers (Kontunen-Soppela et al., unpublished results). The different method of exposure, as well as different species and timing of sampling, may cause the discrepancy in results.

In regard to genes that were upregulated, there was no overlap in the elevated [O_3] alone and in the combined elevated [CO_2] + [O_3] treatment. ROS-related defense reactions were not activated in combined elevated [CO_2] + [O_3], which may result from reduced O_3 uptake (Riikonen et al., 2008a) and therefore diminished need for oxidative stress defense. Since plants possess a range of mechanisms for ROS scavenging during O_3 exposure (Dizengremel et al., 2008; Overmyer et al., 2008), different means for ROS detoxification may occur under better carbon availability (i.e. elevated [CO_2]). For some genes, combined elevated [CO_2] + [O_3] caused changes in GE that were not apparent for either of the gases alone. For instance, GE for genes coding glycolysis-related functions increased, indicating the requirement of energy, enhanced reducing power, and carbon skeletons for biosynthetic pathways. In July GE of some enzymes in the biosynthesis of steroids leading to farnesyl-PP and phytyl-PP was increased, whereas the genes leading to pre-squalene and squalene synthesis decreased in expression. Squalene is precursor of sterols and triterpenoids related to the epicuticular wax formation and its concentration increased in *B. pendula* leaves due to elevated [O_3], whereas concentrations of chlorophyll-related phytol derivatives decrease (Kontunen-Soppela et al., 2007). Therefore, these pathways seem to play a role when plants adapt to increasing concentration of greenhouse gases.

The similar effects of elevated [O_3] and combined treatment on GE were more noticeable in July–August, than in September. Many genes that were related to regulation of GE and protein synthesis, as well as transcription factor GE decreased in the combined treatment in both August and September, correspondingly to the elevated [CO_2], although the GE behind this phenomenon was not alike. Since net photosynthesis in the combined elevated [CO_2] + [O_3] treatment increases parallel to the elevated [CO_2] in midsummer (Riikonen et al., 2008a), the increased carbon uptake may help to maintain the leaf metabolism and delay the senescence in comparison to the elevated [O_3] exposure alone.

5. Conclusions

With the clustering techniques used, we were able to detect similar GE patterns within sampling times and treatments, and to produce meaningful biological information behind the microarray data. Elevated [O_3] reduced photosynthesis and carbon assimilation and induced defensive reactions to oxidative stress resulting in earlier leaf senescence. Transport and proteolysis genes were activated, indicating that at least some remobilization of nutrients for storage was completed. Changes in GE were smaller under elevated [CO_2] as compared to [O_3] treatment, and reflected mainly the surplus of carbon that was directed to synthesis of secondary compounds. The combined elevated [CO_2] + [O_3] treatment resembled the [O_3] treatment, indicating that elevated [CO_2] is not able to totally alleviate the harmful effects of elevated [O_3]. Some GE changes were specific to the combined elevated [CO_2] + [O_3] treatment, for example showing differential expression of steroid biosynthesis genes. This result emphasizes the fact that experiments with O_3 or CO_2-exposure alone are not sufficient to predict plant responses to these gases together, and that field experiments with multiple variables are pivotal in order to understand responses to future environmental conditions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.envpol.2009.10.008.

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