

Gene expression patterns of trembling aspen trees following long-term exposure to interacting elevated CO₂ and tropospheric O₃

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Summary

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- Expression of 4600 poplar expressed sequence tags (ESTs) was studied over the 2001–2002 growing seasons using trees of the moderately ozone (O₃)-tolerant trembling aspen (*Populus tremuloides*) clone 216 exposed to elevated CO₂ and/or O₃ for their entire 5-yr life history.
- Based on replication of the experiment in years 2001 and 2002, 238 genes showed qualitatively similar expression in at least one treatment and were retained for analysis. Of these 238 genes, 185 were significantly regulated (1.5-fold) from one year to the other in at least one treatment studied. Less than 1% of the genes were regulated 2-fold or more.
- In the elevated CO₂ treatment, relatively small numbers of genes were up-regulated, whereas in the O₃ treatment, higher expression of many signaling and defense-related genes and lower expression of several photosynthesis and energy-related genes were observed. Senescence-associated genes (SAGs) and genes involved in the flavanoid pathway were also up-regulated under O₃, with or without CO₂ treatment. Interestingly, the combined treatment of CO₂ plus O₃ resulted in the differential expression of genes that were not up-regulated with individual gas treatments.
- This study represents the first investigation into gene expression following long-term exposure of trees to the interacting effects of elevated CO₂ and O₃ under field conditions. Patterns of gene-specific regulation described in this study correlated with previously published physiological responses of aspen clone 216.

Key words: aspen, expressed sequence tags (ESTs), free-air carbon dioxide enrichment (FACE), global gene expression patterns, macroarrays.

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Introduction

Global increases in air pollutants are major threats to the functioning, structure and diversity of natural and seminatural ecosystems (Bobbink, 1998). Concentrations of the greenhouse gases carbon dioxide (CO₂) (Keeling *et al.*, 1995) and ozone (O₃) (Fowler *et al.*, 1999; Ryerson *et al.*, 2001) are increasing rapidly. Elevated atmospheric CO₂ in short-term exposures has been shown to increase photosynthesis (Drake *et al.*, 1997;

Will & Ceulemans, 1997; Tjoelker *et al.*, 1998a; Noormets *et al.*, 2001a,b), decrease respiration (Volin & Reich, 1996), and stimulate above-ground (Norby *et al.*, 1999) and below-ground (King *et al.*, 2001) growth. Trees grown under elevated CO₂ generally have lower nitrogen concentrations in their foliage, lower rubisco concentrations (Moore *et al.*, 1999), and altered defense compounds (Lindroth *et al.*, 1993, 1997; Wustman *et al.*, 2001). In long-term exposures, the effects of CO₂ are often decreased (Rey & Jarvis, 1998; Tissue *et al.*, 1999) as a

result of reduced sink strength (Gesch *et al.*, 1998) or nutrient-limited habitats (Bryant *et al.*, 1998; Oren *et al.*, 2001). Elevated CO₂ has been reported to decrease the concentration of antioxidants such as glutathione and ascorbate (Schwanz *et al.*, 1996; Polle & Pell, 1999; Wustman *et al.*, 2001), but no effect of elevated CO₂ on antioxidant transcripts has also been reported (Schwanz & Polle, 1998; Sehmer *et al.*, 1998).

Tropospheric O₃ is one of the most ubiquitous and damaging phytotoxins known (Broadmeadow, 1998). Ozone affects plant growth by reducing stomatal conductance, degrading rubisco and chlorophyll, decreasing photosynthesis and increasing photorespiration, inducing premature senescence and delaying bud break (Landry & Pell, 1993; Volin & Reich, 1996; Nali *et al.*, 1998). Ozone enters mesophyll tissue through stomata and produces free hydroxyl radicals (OH), superoxide anions (O₂ ·⁻) and hydrogen peroxide (H₂O₂). This results in peroxidation and denaturation of cellular membranes (Wellburn & Wellburn, 1996; Ederli *et al.*, 1997; Pell *et al.*, 1997; Loreto & Velikova, 2001), leading to an accelerated foliar senescence through induction of ethylene, and changes in specific mRNAs (Glick *et al.*, 1995) as reported in many species (Nie *et al.*, 1993; Pell *et al.*, 1997; Miller *et al.*, 1999). Ozone induces hypersensitive stress responses (HRs) leading to apoptosis, which is coordinated by the signaling pathways of ethylene and jasmonic acid (Rao & Davis, 1999; Rao *et al.*, 2000). Reactive oxygen species (ROSs) induced by O₃ are scavenged in different subcellular compartments by antioxidant enzymes. Therefore, plants undergo numerous adjustments to counteract the effects of stress by regulating a number of genes involved in stress responses (Reymond *et al.*, 2000; Cheong *et al.*, 2002). Several studies have shown that O₃ tends to increase expression of defense response genes such as glutathione S-transferase (GST), phenylalanine ammonia-lyase (PAL), and a cytosolic copper/zinc (Cu/Zn) superoxide dismutase (SOD) (Akkapeddi *et al.*, 1999; Wustman *et al.*, 2001). Photosynthetic protein mRNAs (Conklin & Last, 1995) and polyamines (Scalet *et al.*, 1994) are also commonly up-regulated by O₃. Ozone alters the thylakoid and chloroplast membranes in turn, decreasing photosynthetic capacity (Tognini *et al.*, 1997) and weakening plasma membranes (Tokarska-Schlattner *et al.*, 1997).

In the future, elevated tropospheric O₃ will co-occur in the atmosphere with elevated atmospheric CO₂ over large areas of the world's forests. Many studies have shown that elevated CO₂ reduces the adverse effects of elevated O₃ (Volin & Reich, 1996; Booker *et al.*, 1997; Grams *et al.*, 1999), while other studies have shown that elevated CO₂ exacerbates the negative effects of elevated O₃ (Kull *et al.*, 1996; Lutz *et al.*, 2000; McDonald *et al.*, 2002; Percy *et al.*, 2002). Very little is known about the impacts of these two greenhouse gases in combination on gene expression. However, Wustman *et al.* (2001) reported that CO₂ did not ameliorate the harmful effects of O₃. In aspen clones exposed to the co-occurring gases, increased damage to chloroplasts and suppression of the ascorbate-glutathione pathway were found (Wustman *et al.*, 2001).

The impacts of elevated tropospheric O₃ and elevated atmospheric CO₂, alone or in combination, are being studied at the Aspen free-air carbon dioxide enrichment (FACE) project near Rhinelander, Wisconsin, USA. At this facility (Dickson *et al.*, 2000), we have been exposing an aggrading northern forest ecosystem consisting of trembling aspen (*Populus tremuloides* Michx.), paper birch (*Betula papyrifera* Marsh.) and sugar maple (*Acer saccharum* Marsh.) to elevated CO₂ and O₃, alone and in combination, for the past 5 yr (Karnosky *et al.*, 2003). For this study, we examined aspen clone 216, which is moderately O₃-tolerant (Isebrands *et al.*, 2001). Most previous studies of gene expression have been carried out on samples collected from the glasshouse or chamber with short-term exposures to either elevated CO₂ or elevated O₃ alone. In our study, we collected samples from trees that had grown under elevated CO₂ and O₃ over their entire 5-yr life histories.

The objective of this study was to examine the effects of elevated CO₂ and/or O₃ on global gene expression in aspen. Furthermore, we sought to detect linkages between gene expression and documented physiological responses following long-term exposure to elevated CO₂ and O₃, alone or in combination. We have shown that global gene expression analyses can be performed on field-grown trees and that these data can be linked to whole-tree responses.

Materials and Methods

The FACE experiment and biological materials

The Aspen FACE facility is located at the United States Department of Agriculture (USDA) Forest Service, Harshaw experimental farm near Rhinelander, WI, USA (Karnosky *et al.*, 1999, 2003; Dickson *et al.*, 2000). The experimental site consists of four rings each of control (ambient air; CO₂ concentration 360 ppm), elevated CO₂ (560 ppm), elevated O₃ (1.5 times ambient) and elevated CO₂ plus elevated O₃ conditions in triplicate rings of 30-m diameter each. The eastern half of each ring consists of a randomized mixture of two-tree plots of five aspen clones including the relatively O₃-tolerant clone 216 (Isebrands *et al.*, 2001) used in this study. The remaining half of each ring is divided into two sections with either alternating paper birch and aspen or alternating sugar maple and aspen. Detailed information about the planting design, location and gas exposure in the Aspen FACE study is given in Karnosky *et al.* (1998, 1999, 2002) and Dickson *et al.* (2000).

Young, expanding aspen leaves of leaf plastochron index (LPI) 1–5 (Larson & Isebrands, 1971) were collected in August of 2001 and 2002 from the O₃-tolerant clone 216 from the Aspen FACE facility. Leaves were harvested from 10:00 to 12:00 hours and samples were pooled from six trees from each of the three replicate rings from the FACE project. Leaves were wrapped in aluminum foil and immediately placed in liquid nitrogen before being taken to the laboratory, where they were transferred to –80°C storage until further use.

Generation of cDNA array

A cDNA array was designed from selected expressed sequence tags (ESTs) from adventitious roots (library R, 3984 ESTs) and leaves (library F, 486 ESTs) described in Kohler *et al.* (2003). The selected ESTs corresponded to single sequences or to ESTs representative of contigs of at least four ESTs (Kohler *et al.*, 2003). Assembly of the individual ESTs into groups of tentative consensus sequences representing unique transcripts was performed using the contig routine (80% identity over a 40-nt length) of SEQUENCHER (version 3.1.1 for Macintosh; Gene Codes Corporation, Ann Arbor, MI, USA). A set of negative control spots corresponding to the cloning vector (four replicates of a pTriplex plasmid sequence and 12 replicates of a pTriplex polylinker sequence; BD Biosciences, Palo Alto, CA, USA), 17 replicates of a human desmine cDNA (kindly provided by T. Desprez, INRA Versailles, France), and 25 and 29 cDNAs encoding different genes from, respectively, fungal cDNA libraries of *Pisolithus tinctorius* and *Laccaria bicolor* (Peter *et al.*, 2004) were also placed on the array along with 35 spot locations without nucleic material (water). All cDNAs from the root and leaf libraries were amplified from overnight culture of bacteria by polymerase chain reaction (PCR) and purified on the Multiscreen PCR system (Millipore, Molsheim, France), and the purity and length of all PCR products were checked by agarose gel electrophoresis. cDNA inserts (30–50 ng μl^{-1}) were single-spotted onto positively charged nylon membranes by using the BioGrid arrayer (BioRobotics, Cambridge, UK) according to the array manufacturer's instructions (Eurogentec, Saraing, Belgium). The 0.4- μm pins of the 384-pin gadget deposited 100 nl of each PCR product and the final density of spots on the array was about 66 cDNA cm^{-2} . Hybridization with radiolabeled probes corresponding to the plasmid polylinker of the plasmid vector used for construction of R and F libraries was routinely performed to ensure quality of cDNA arraying on filters.

RNA isolation, probe synthesis and hybridization

Total RNA was extracted from pooled leaf samples collected from six trees in each treatment, using a modified Chang *et al.* (1993) method. Polyvinylpolypyrrolidone (PVPP insoluble) was added to increase the purity of the RNA yield. Complex cDNA probes were then prepared by reverse transcription using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and the SMART-PCR cDNA synthesis kit (BD Biosciences) and hybridized to cDNA arrays as described in Duplessis *et al.* (2005). Different sets of arrays were used for hybridization of year-replicate samples and of samples from different conditions. Pooling of leaf samples from different trees from each FACE ring helped to minimize the variation among individual samples, arrays and probes.

Data analysis

The 16-bit TIFF images obtained with the phosphorimager imaging system were imported into the X-DOT READER program (version 2.0; COSE, Paris, France) and quantified. Detection and quantification of the 4600 signals representing hybridized DNA (each cDNA plus appropriate controls duplicated) were performed using the 'volume quantification' method. Each spot was defined by automatic grid positioning over the array image and the average pixel intensity of each spot was determined. Net signal was determined by subtraction of global background measured all over the array from the intensity for each spot. Spots deemed not suitable for accurate quantification because of array artifacts were flagged and excluded from further analysis. The data table generated by X-DOT READER, containing the intensity of each spot, was then exported to the Excel X:mac worksheet program (Microsoft Corporation, Redmond, WA, USA) for further manipulation. Spots that had an intensity of less than twice that of the background were flagged as undetectable and had their intensity raised to a minimum threshold value of 0.1 to avoid spurious expression level ratios at the bottom of the spot intensity range.

To take account of experimental variations in the specific activity of the cDNA probe preparations or in exposure time that might alter the signal intensity, the raw data obtained from different hybridizations were normalized (scaled) by dividing the intensity of each spot by the average of the intensities of all the spots present on the filter, to obtain a centered, normalized value (Eisen *et al.*, 1998). Then we calculated a 'flag limit' corresponding to the remaining intensity measured at the locations of empty spots (water) on nylon arrays. This limit corresponded to the sum of the mean and the standard error calculated from signals measured at the 35 empty spot locations. We flagged and then separated from the data set all the spots that showed intensity lower than 2-fold the 'flag limit' in all conditions (both years 2001 and 2002 in control, CO_2 , O_3 and combined CO_2/O_3 treatments). This filtering process allowed separation of clones close to the background noise level on the array from the analysis. The Microsoft Excel software was finally used to calculate the expression level ratios for remaining genes on arrays for all treatments ($\text{O}_3/\text{control}$; $\text{CO}_2/\text{control}$; combined $\text{CO}_2 + \text{O}_3/\text{control}$) in each year-replicate (2001 and 2002).

Data quality assessment was performed using analysis of variance (*t*-test) and a Bayesian statistical framework implemented in the Cyber-T web interface (<http://visitor.igb.uci.edu/genex/cybert/>) (Long *et al.*, 2001; Baldi & Hatfield, 2002). Based on the statistical analysis, a gene was considered significantly up- or down-regulated if the *t*-test *P*-value was lower than 0.05 in at least one treatment (CO_2 or O_3 or combined CO_2/O_3) and the mean ratio associated with this is above a 1.5-fold limit. For the final analysis, the fold changes of gene expression in year-replicates were averaged and genes with expression falling between 0 and 1 were multiplied by -1 and inverted to facilitate their interpretation. Microsoft Excel spreadsheets with

the remaining genes are available in Table S1 (available online as supplementary material) and at the website <http://mycor.nancy.inra.fr/poplarDB/pub>. Excel spreadsheets with raw data and associated statistical results are also available at the same locations (CO₂ vs control, Table S2; O₃ vs control, Table S3; CO₂ + O₃ vs control, Table S4). Cyber-T includes a computational method (Posterior Probability of Differential Expression (PPDE)) for estimating experiment-wide false-positive and false-negative results based on the modeling of *P*-value distributions (Baldi & Hatfield, 2002), and results for these estimations are given in the two last columns of Tables S3 and S4. These columns are missing in Table S2 (CO₂ conditions), where the PPDE calculation was impossible because of the data distribution in CO₂ treatment replicate data sets.

Fold changes were supplied to the GENECLUSTER 2 program (<http://www.broad.mit.edu/cancer/software/genecluster2/gc2.html>) to identify groups of genes having similar patterns of regulation under the different treatments (Tamayo *et al.*, 1999). In GENECLUSTER 2, the numbers of self-organizing map (SOM) rows and SOM columns were set to four and four, respectively, to explicitly build a SOM with 16 clusters (see Fig. 2 below). The number of iterations was set to 500 000 and the default parameters were used for the other settings. We only considered clusters with genes and removed those that remained empty (see Fig. 2 below). The calculation allowed us to derive genes with similar expression patterns without regard to the magnitude of the ratios. All descriptions of biological materials and procedures and raw data will be posted on the mycor website (<http://mycor.nancy.inra.fr/poplarDB/pub.html>) through public access to a BASE DB in construction which will comply with all MIAME (Minimum Information About a Microarray Experiment) standards set for microarray data (Brazma *et al.*, 2001).

RNA blot analyses

For RNA blot analyses, electrophoresis under denaturing conditions was performed with 1.2% agarose containing 0.7 M formaldehyde (Lehrach *et al.*, 1977) with RNA sampled in the

year 2002. Gels were stained with ethidium bromide and blotted on nylon membranes (Hybond-N+; Amersham Pharmacia Biotech, Piscataway, NJ, USA) as described by the manufacturer. Hybridization was carried out as recommended by Amersham Pharmacia Biotech and signals were revealed by autoradiography using the Bio-Rad FX phosphorimager (Bio-Rad, Hercules, CA, USA).

Results

FACE project and experimental design

In order to investigate the effects of elevated tropospheric O₃ and elevated atmospheric CO₂, alone or in combination, at the molecular level, we have studied gene expression in trembling aspen exposed to these treatments in the FACE project near Rhinelander, Wisconsin, USA. In this facility, among other northern forest trees, trembling aspens (*P. tremuloides*) were exposed to elevated CO₂ and O₃, alone and in combination, for the 5 yr before this study (Karnosky *et al.*, 2003). In this study, we particularly examined the aspen clone 216, which is moderately O₃-tolerant (Isebrands *et al.*, 2001). Samples were taken in the years 2001 and 2002 from LPI 1–5 from six trees in each of the three replicate rings of the FACE project and pooled by treatments in each year. The experimental design for this gene expression study consisted of two 'year-replicates' of control conditions and the three considered treatments (elevated CO₂, elevated O₃ and combined elevated CO₂ and O₃).

Gene expression profiles in aspen leaves

To examine the gene activity changes associated with the long-term effects of exposure of aspen leaves to elevated CO₂ and O₃, alone or in combination, we performed a large-scale expression analysis using a nylon cDNA array. Radioactive [³³P] cDNA probes were prepared from replicate sets (years 2001 and 2002) of RNA extracted from aspen leaves exposed to elevated concentrations of CO₂ and O₃, as shown in Table 1. Radiolabeled probes were incubated with 4608-element nylon

Pollutant	Year				
	1998	1999	2000	2001	2002
CO ₂ ¹ (control and + O ₃)	360	360	350	365	370
CO ₂ ¹ (+ CO ₂ and + CO ₂ + O ₃)	530	548	545	505	504
O ₃ ² (control and + CO ₂)	34.6	36.9	34.5	36.6	33.1
O ₃ ² (+ O ₃ and + CO ₂ + O ₃)	54.5	51.7	48.1	52.8	49.0
O ₃ ³ seasonal dose (control and + CO ₂)	59.1	62.8	55.8	61.6	50.6
O ₃ ³ seasonal dose (+ O ₃ and + CO ₂ + O ₃)	97.8	89	79.9	90.7	80.8
Seasonal exposure duration (d)	166	143	139	143	138

Table 1 Summary of treatment exposures for the five years in which aspen (*Populus tremuloides*) trees were exposed to elevated CO₂ and O₃, alone and in combination, at the Aspen FACE facility

¹Daytime average (ppm).

²Daytime mean concentration (ppb).

³Sum 0 (ppmh).

cDNA arrays. These cDNAs correspond to *Populus trichocarpa* Torr. and Gray \times *P. deltoides* Barrtr. ESTs from root (3984 ESTs) and leaf (486 ESTs) libraries previously described (Kohler *et al.*, 2003). These clones encompass a large array of metabolic processes and response elements to biotic and/or abiotic stresses. In order to avoid as much as possible non-specific hybridization signals between labeled aspen cDNA probes and poplar hybrid cDNA targets on arrays, our procedure included stringent washing conditions after hybridizations (Duplessis *et al.*, 2005).

To test the quality of reproducibility between replicates sampled in the years 2001 and 2002, we hybridized sets of cDNA arrays with radioactive cDNAs prepared from RNA extracted during the years 2001 and 2002 and compared expression levels (normalized) for each treatment (Fig. 1). The Pearson correlation coefficient of 0.92 calculated for the control conditions indicated reasonably good reproducibility between the years 2001 and 2002. However, Pearson correlation coefficients measured for the treatments with elevated CO₂ and O₃, alone or in combination, were lower, from 0.67 (elevated O₃) to 0.88 (combined elevated CO₂ and O₃), indicating less reproducible levels of expression for a large proportion of the genes plotted. This low correlation was expected for samples from trees grown in field conditions.

We applied a filter to our data set based on the intensity of the background signal measured at empty spot locations on arrays (nonspecific hybridization). We flagged clones that were not above this limit in all treatments and in control conditions and separated them from the analysis. This flag limit was stringent enough to allow us to eliminate ESTs from the data set associated with low signal values for various reasons (e.g. sequence divergence between poplar species or tissue specificity of cDNA targets). This resulted in a final data set of 801 poplar ESTs after filtering. This relatively small number of ESTs, when compared with the 4608 initial ESTs spotted on the cDNA array, might be an expression of the sequence divergence between the two poplar species used in this study. However, a recent analysis of ESTs from different species showed little variation at sequence level between cDNAs from different poplar species (Sterky *et al.*, 2004). Similar findings were obtained during heterologous array analyses between spruce and pine (van Zyl *et al.*, 2002; Stasolla *et al.*, 2004). The subset number of ESTs eliminated from the present data set may reflect a combined effect of the specificity of an important fraction of root cDNA targets on the array during hybridization with leaf cDNA probes and wash stringency after hybridization. Several experiments performed with the same poplar cDNA array hybridized with probes extracted from both leaf and root tissues have shown heterogeneity in signal intensities based on tissue specificity (A. Kohler, INRA, Nancy, France, pers. comm.). Moreover, the stringency during washes following hybridization could also have an important impact on the mean signal measured on a cDNA array (van Zyl *et al.*, 2002). In the present study, this point is

underlined by the percentages of root and leaf ESTs that survived the filtering step (12% and 66%, respectively) in the final data set. From among the 801 ESTs that were above the flag limit, we distinguished genes that were significantly regulated in at least one treatment between replicates sampled in the years 2001 and 2002 using analysis of variance (*t*-test) and the Bayesian statistical framework implemented in the Cyber-T web interface (ln *P*-value = 0.05; Tables S2, S3 and S4) (Long *et al.*, 2001; Baldi & Hatfield, 2002). We then determined the level of regulation of gene expression by dividing the normalized mRNA concentration measured in the three treatments by the normalized mRNA concentration measured in control conditions in the years 2001 and 2002. Of the 801 ESTs, 238 showed significantly similar patterns of expression in at least one treatment: 20 in the elevated CO₂ treatment between year-replicates, 167 in the elevated O₃ treatment and 111 in the combined elevated CO₂ and O₃ treatment. We then calculated the mean expression ratio of the 238 selected ESTs in each treatment and flagged the genes that showed qualitatively different expression patterns between year-replicates (Table S1). From among the ESTs with significant levels of expression, we distinguished those that showed a level of regulation of at least 1.5-fold and selected 185 ESTs that were up- or down-regulated in at least one of the treatments: three (two up- and one down-regulated) in the case of the elevated CO₂ treatment; 95 (88 up- and seven down-regulated) in the case of the elevated O₃ treatment and 51 (44 up- and seven down-regulated) in the case of the combined elevated CO₂ and O₃ treatment. The relatively small number of genes showing a significant regulation profile between 2001 and 2002 in the elevated CO₂ treatment highlights once again the high variability in this treatment and the differences affecting expression in leaves between the two years, when compared to the numbers of significantly regulated genes in the two other treatments. The Cyber-T procedure allowed identification of false positives within calculated *P*-values. This calculation was not possible for the CO₂ conditions because of the variability in the data distribution between the 2001 and 2002 year-replicates (see PPDE column in Tables S2, S3 and S4). For the O₃ and combined CO₂ and O₃ treatments, only less than 1% of the genes, in one case as in the other, were regulated by a 2-fold level or more (Table 2). If regulation levels did not exceed 3-fold in the mean ratios calculated, the highest regulation levels measured for 2001 or 2002, considered separately, reached 12-fold (O₃ treatment in 2001). Another important observation here is the small number of genes down-regulated in the three treatments compared with control conditions (less than 5% of significant genes in each treatment).

The reduced number of ESTs showing consistent expression in the 2001 and 2002 year-replicates could reflect a true environmental effect of the treatments on aspen clones in the FACE facility. We also observed quantitative differences between the years 2001 and 2002 rather than qualitative changes in regulation of gene expression. This observation

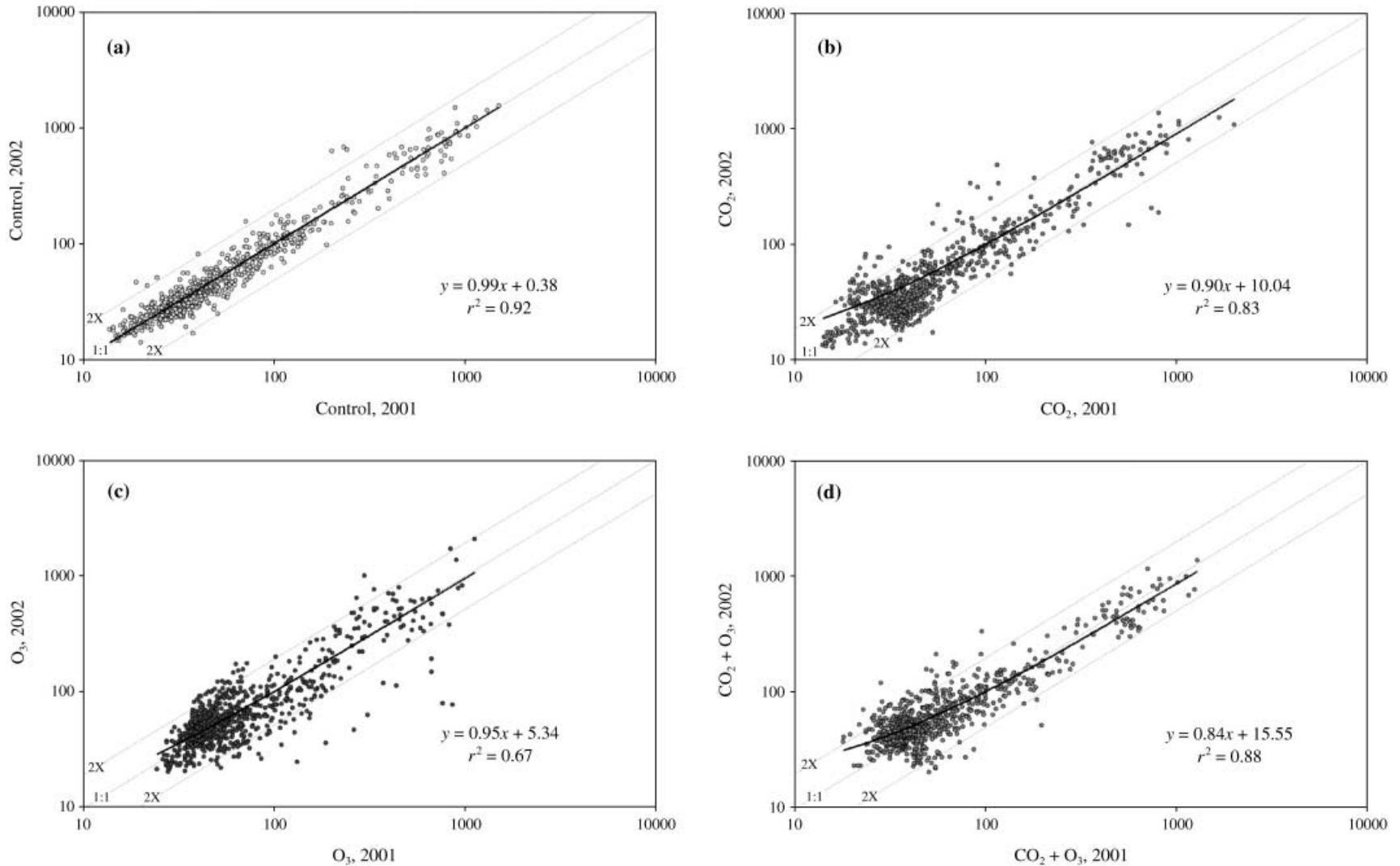


Fig. 1 Scatter plots comparing the signal intensities of pairs of arrays hybridized with two sets of cDNA probes prepared from RNA extracts from two year-replicates (2001 on the x -axis and 2002 on the y -axis). (a) Normalized mRNA abundance of 801 expressed sequence tags (ESTs) measured in leaves of aspen (*Populus tremuloides*) grown in control conditions. (b) Normalized mRNA abundance of 801 ESTs measured in leaves of aspen grown under elevated CO₂ concentration. (c) Normalized mRNA abundance of 801 ESTs measured in leaves of aspen grown under elevated O₃ concentration. (d) Normalized mRNA abundance of 801 ESTs measured in leaves of aspen grown under combined elevated CO₂ and O₃ concentrations. All axes show a log₁₀ scale and arbitrary units. On each scatter plot, dotted lines indicate a 2-fold difference between year-replicates and the 1 : 1 ratio line. A linear regression equation and correlation factor r^2 are given for each year-duplicate comparison.

Table 2 Regulation of gene expression for selected significantly regulated genes in leaves of aspen (*Populus tremuloides*) grown under elevated CO₂, elevated O₃ and combined elevated CO₂ and O₃ concentrations compared with control conditions (C)

Clone ID	Accession no.	Gene function	CO ₂ /C	ln <i>P</i> -value	O ₃ /C	ln <i>P</i> -value	O ₃ + CO ₂ /C	ln <i>P</i> -value	SOM cluster	RNA blot
F02G09	CA820757	Hypothetical protein	1.76	0.01229	1.59		1.46	0.03878	c12	
R18B06	CA822984	Cysteine protease	1.37	0.01332	1.88	0.04904	1.79	0.03431	c12	*
R75F07	CA826280	ACC oxidase	-1.22	0.00952	1.27	0.02943	1.86		c11	*
RA01F02	CA821251	Ribulose-5-phosphate-3-epimerase	-1.43	0.04338	1.07		-1.08		c4	
R74G09	CA826232	Aquaporin	-1.58	0.0238	1.07		1.02		c8	
R10A04	CA822570	Wound-induced protein	1.38		2.57	0.0338	1.89		c13	*
R04C10	CA822148	Calmodulin 1	1.29		1.94	0.04808	1.62	0.00365	c12	
R12E11	CA822712	Sucrose synthase	1.22		1.91	0.04547	1.62		c12	
R27H05	CA823548	Ubiquitin/ribosomal protein S27a	1.14		1.87	0.02579	1.74		c12	
R08B12	CA822430	Zinc-finger protein	1.27		1.86	0.00712	1.62	0.02152	c12	
R33H02	CA823880	Histone H4	1.56		1.84	0.026	2.07		c13	
R06C05	CA822298	Histone H3	1.41		1.84	0.00295	1.95		c13	
R22D07	CA823207	Ribosomal protein L8	1.17		1.82	0.00762	1.56	0.0069	c9	
R25F07	CA823402	Inorganic pyrophosphatase	1.21		1.81	0.02828	1.39	0.04062	c9	
R21B12	CA823125	ATP-citrate lyase	1.37		1.81	0.0033	1.66	0.03525	c12	
F11C02	CA821163	60S acidic ribosomal protein L12	1.09		1.8	0.03842	1.43	0.01225	c9	
R10G01	CA822614	Kinetochore-associated protein Skp1	-1.03		1.8	0.01635	1.36	0.0438	c8	
R03D06	CA822075	Alcohol dehydrogenase	-1.01		1.78	0.02634	1.31		c8	
R02H08	CA822034	Glutamine synthetase	-1.07		1.69	0.04107	1.4		c8	
F11A19	CA821143	Rubisco small subunit	-1.27		-1.98	0.00052	-1.47	0.03338	c0	*
R71C08	CA826021	Aquaporin	-1.18		1.55		2.46	0.0153	c11	
R77F06	CA826403	Formate dehydrogenase	-1.12		1.99		2.36	0.04624	c11	
R75C04	CA826255	G3PDH	-1.17	0.02355	1.49	0.01327	2.34	0.00628	c11	
R46C01	CA824657	POP3 wound-induced protein	1.07		1.68	0.0078	2.22	0.0003	c13	*
R57F07	CA825362	Naringenin-chalcone synthase	1.02		1.47		2.11	0.04185	c13	
R57C09	CA825341	Mitochondrial phosphate transporter	-1.31		1.43		2.05	0.04031	c11	
R73E09	CA826154	Dehydrin	-1.04		1.45		1.97	0.0112	c11	
R20E04	CA823085	Beta tubulin 1	1.3		1.65	0.04973	1.91	0.02682	c13	
R20A02	CA823050	Elongation factor eF-2	1.07		1.48		1.91	0.04272	c10	
F11B12	CA821155	Thaumatin-like protein	1.63		1.63		1.87	0.03375	c12	
R17C05	CA822967	Hypothetical protein	-1.02		1.72	0.02518	1.77	0.01793	c11	
R25G01	CA823406	S-adenosylmethionine synthetase	1.2		1.35		1.77	0.03061	c10	*
R16G02	CA822945	Alpha-6 tubulin	1.33		1.63	0.01647	1.75	0.03363	c12	
R16G03	CA822946	Xyloglucan endotransglycosylase	1.78		1.44	0.01327	1.75	0.0078	c12	
R77F05	CA826402	Polyubiquitin	1.49		1.99		1.71	0.04684	c12	
F11B16	CA821159	Carbonate dehydratase	1.02		-1.37		-1.82	0.03151	c1	
F02E05	CA820743	Rubisco small subunit	-1.34		-1.78	0.00123	-1.88	0.04585	c0	*

Associated ln *P*-values < 0.05 as calculated by the Cyber-T program between year-replicates 2001 and 2002 are given along with mean ratios. These genes were selected from Table S1 for their high level of regulation of expression. Rows are sorted by level of regulation measured in the three different treatments. Signal ratios < 1.0 were inverted and multiplied by -1 to aid their interpretation. Mean ratios highlighted in gray indicate genes that showed similar expression patterns in years 2001 and 2002. The self-organizing map (SOM) cluster column indicates the corresponding cluster in Fig. 2. The asterisk indicates genes tested by RNA blots. ACC oxidase, 1-aminocyclopropane-1-carboxylate oxidase.

may be a result of sampling effects and environmental conditions in the hours or days before sampling. These quantitative differences, which could be caused by another stress (biotic or abiotic) in one year and not the other, complicate the analysis but also strengthen the validation of the significant regulation of gene expression detected for the different treatments in the years 2001 and 2002. For example, there was some infestation by tent caterpillars at the FACE site and this may have had some effect on defense gene expression levels, even in control

rings. This biotic stress may also have partly accounted for the small number of significant genes found in some of the treatments (e.g. CO₂).

Cluster analysis of gene expression

We grouped the expression profiles of the 238 significantly regulated ESTs in the elevated CO₂ and/or O₃ treatments (alone or in combination) with the program GENECLUSTER

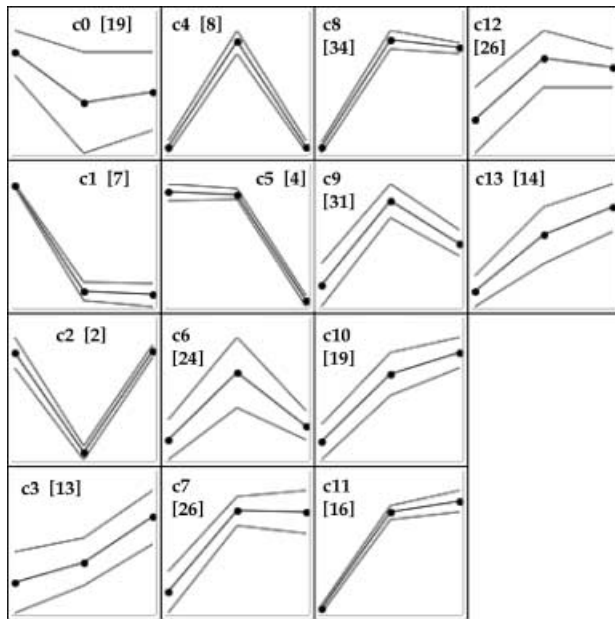


Fig. 2 Self-organizing map (SOM) clusters of expression profiles measured in leaves of aspen (*Populus tremuloides*) grown under elevated CO₂, elevated O₃ and combined elevated CO₂ and O₃ concentrations for genes that showed significant regulation of gene expression in at least one treatment when compared to the control conditions. Each graph displays the mean pattern of expression of the transcripts in that cluster (lines with solid circles) and the standard deviation of average expression (lines with no solid circles). The number of transcripts [expressed sequence tags (ESTs)] in each cluster is given in each SOM. The x-axis represents normalized gene expression and the y-axis the ratios measured in the three treatments compared with control conditions. The ESTs in each cluster are identified in Table S1 and mentioned in Table 2. Cluster number and number of genes in that cluster are given in each panel.

2. This allowed us to recognize features in complex, multidimensional gene expression data and classify them into groups of genes sharing similar patterns of regulation in different treatments (Tamayo *et al.*, 1999). Figure 2 presents the 12 clusters derived by GENECLUSTER 2 (using a 4 × 4 grid with 16 seeds and 500 000 iterations), and the full list of genes included in each cluster is given in Table S1. As a consequence of the low regulation observed in the CO₂ treatment, most of the clusters corresponded to genes more highly expressed under elevated O₃ alone (cluster (c) 4, c6 and c9) or in combination with CO₂ (c3, c7, c8, c10, c11, c12 and c13). Of the significantly regulated ESTs, only a few showed an up- or down-regulated profile in elevated CO₂, alone or in combination with O₃, when compared with the treatment with elevated O₃ alone. This last point underlines the substantial impact of elevated O₃ alone, even on O₃-tolerant aspen clone 216, in the long term. The various SOM profiles could be reduced to a limited set of expression patterns based on the graphical outputs. For example, SOMs c0 and c1 could be grouped, and, as presented in Table S1, these clusters consisted of ESTs encoding similar functions. Occurrences

of different cellular functions could be detected in other clusters (e.g. c7, c8 and c11), but most of the clusters shared a similar pattern, which corresponded to higher expression in elevated O₃, alone or combined with CO₂, than in elevated CO₂ alone.

Table 2 presents a subset of the most up- or down-regulated genes (1.5-fold regulation) presented in Table S1 in at least one treatment compared with control conditions in the years 2001 and 2002. Gene expression determined by RNA blot is also indicated in Table 2 and the SOM clusters generated are given in Fig. 2. This subset of genes is representative of the global response observed for the different treatments. Few genes were significantly regulated in the CO₂ treatment, while a much larger number of genes were up- or down-regulated in the O₃ treatment, alone or in combination with CO₂. In the CO₂ treatment, up-regulation of genes encoding a cystein protease and a hypothetical protein was observed, while genes encoding 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase, an aquaporine and ribulose-5-phosphate epimerase were down-regulated. As shown in the SOM clustering profiles, most of the functions altered in expression in the O₃ treatment were similarly altered in the combined treatment with CO₂. Up-regulated functions encoded stress-related proteins such as wound-induced proteins, cytoskeleton elements (alpha and beta tubulin) and the secondary metabolism enzyme naringenin-chalcone synthase. Some important functions for leaf physiology were down-regulated in similar ways in all treatments, such as the rubisco small subunit. Several genes encoding enzymes falling into the same cellular categories as presented in Table 2 were also found in the various treatments, but with lower levels of regulation (< 1.5-fold).

RNA blot analyses

To validate observations obtained using the cDNA array approach we used a traditional molecular method. We selected different genes based on their treatment-specific profiles and tested them by northern blot analysis, which gives a good indication of the transcript abundances in the various treatments (Fig. 3). RNA blot analyses for the rubisco small subunit, cystein protease, POP3 wound-induced protein, Cu/Zn superoxide dismutase, ACC oxidase, S-adenosylmethionine synthetase and wound-induced protein genes correlated with expression levels observed in the cDNA array. Thus, the usefulness of nylon-based cDNA arrays for global gene expression profiling in identifying genes with treatment-specific expression patterns from samples harvested in field conditions was confirmed.

Discussion

Ozone causes an oxidative burst in plants, which in turn produces reactive oxygen species (ROS) and induces a series of signaling pathways leading to apoptosis. Under elevated O₃, a number of ESTs that are involved in signal transduction,

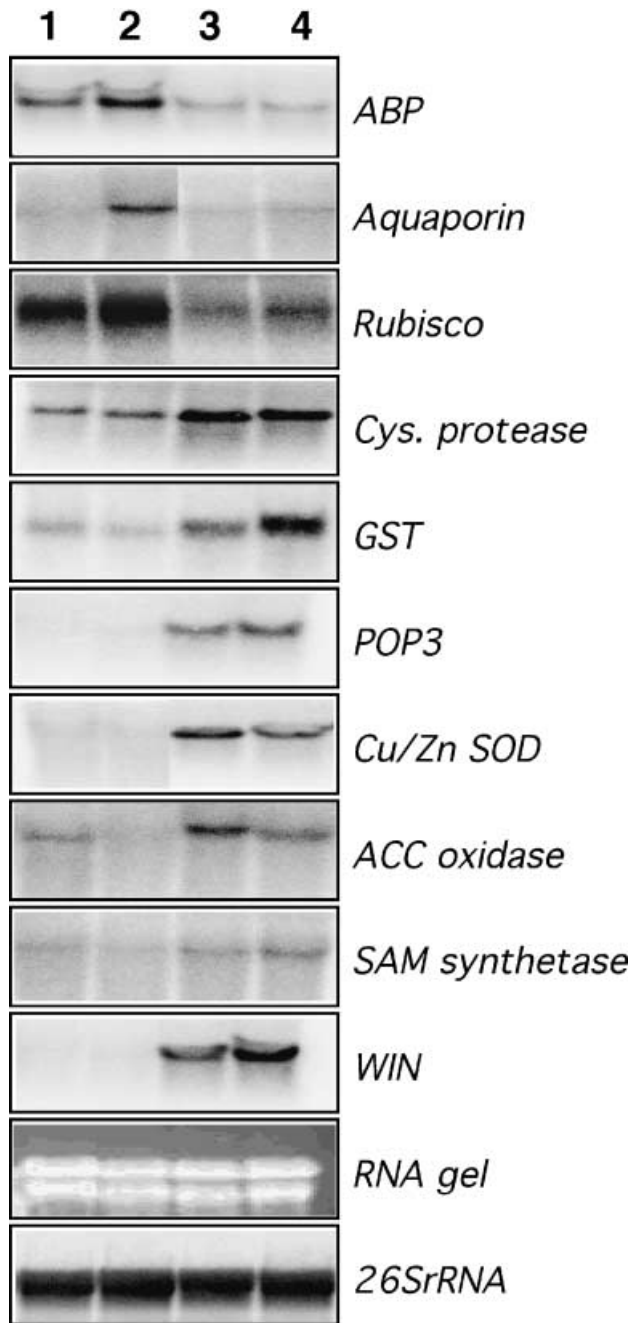


Fig. 3 RNA blot analyses for validation of cDNA arrays. Hybridization of 10 poplar cDNA probes to total RNAs extracted from aspen (*Populus tremuloides*) grown in control (lane 1), elevated CO₂ concentration (lane 2), elevated O₃ concentration (lane 3) and combined elevated CO₂ and O₃ concentration (lane 4) treatments in the year-replicate 2002 is shown. Total RNAs, isolated from leaves of aspen clone 216 grown in the different conditions of the FACE project (Karnosky *et al.*, 2003), were hybridized with selected ³²P-labeled cDNA inserts and the 26S rDNA encoding gene. rRNAs on gel are shown as quality control of electrophoresis. ABP, auxin-binding protein; rubisco, ribulose biphosphate carboxylase/oxygenase small subunit; GST, glutathione S-transferase; POP3, POP3 wound-induced protein; Cu/Zn SOD, copper/zinc superoxide dismutase; ACC oxidase, 1-aminocyclopropane-1-carboxylate oxidase; SAM synthetase, S-adenosylmethionine synthetase; WIP, wound-induced protein.

defense, and the cell cycle, particularly in senescence or cell death, were up-regulated in trembling aspen growing in the FACE facility. These ESTs included wound-induced proteins, proteases, ethylene biosynthesis-related genes such as ACC oxidase, water stress-induced proteins and signaling proteins. ACC oxidase oxidizes ACC to ethylene. Ethylene is a signaling molecule that stimulates processes leading cell death, including senescence, in plants. Generally, in elevated O₃ environments, high concentrations of ethylene are produced (Wolfgang *et al.*, 2002). Senescence-associated genes (SAGs) that were highly expressed in elevated O₃ were cysteine protease (Lohman *et al.*, 1994), glutamine synthetase (Bernhard & Matile, 1994), and chloroplast 30S ribosomal protein S7. The gene encoding the POP3 protein is a wound-inducible gene also related to defense (Van Damme *et al.*, 2002), which was up-regulated under elevated O₃. A thaumatin-like protein-encoding gene was also up-regulated under elevated O₃. These proteins also fall into the plant defense category and are known as pathogenesis-related proteins (S. Duplessis, unpublished).

One gene that was down-regulated gene under elevated O₃ corresponds to auxin-binding protein, which is postulated to be responsible for cell expansion and growth (Jones & Herman, 1993). Many genes involved in photosynthesis were also down-regulated under elevated O₃, including genes coding for rubisco activase, the small subunit of ribulose biphosphate carboxylase oxydase (rubisco), the chlorophyll *a/b* binding protein, and photosystem II (PSII) oxygen-evolving enhancer protein 3. The down-regulation observed for many genes involved in photosynthesis under elevated O₃ may contribute to the reduced photosynthetic rates observed under elevated O₃ (Noormets *et al.*, 2001a,b). In addition, we detected evidence of premature senescence being triggered under elevated O₃ (Sharma *et al.*, 2003).

It is well known that O₃ causes premature foliar senescence and early leaf abscission (Landry & Pell, 1993; Volin & Reich, 1996; Nali *et al.*, 1998). Genes up-regulated under elevated O₃ that we believe to play a role in the early senescence response included defense genes, antioxidant genes, genes involved in photorespiration or signal transduction, genes encoding wound-induced proteins, senescence-associated genes, and genes involved in the phenylpropanoid pathway associated with anthocyanin production (Winkel-Shirley, 2003). It is interesting to note that similar functions were regulated in the transcriptome analysis of the model plant *Arabidopsis* under short-term O₃ exposure (Tamaoki *et al.*, 2003), such as plant defense genes (mainly up-regulated) and energy-related genes (down-regulated).

Under elevated CO₂, up-regulated genes included photosynthetic genes encoding chloroplast 30S ribosomal protein and PSII and photosystem q(b) proteins, important genes for growth, and genes encoding auxin-binding proteins. This suggests that the photosynthetic machinery continues to produce larger amounts of transcripts in aspen trees growing under long-term exposure to elevated CO₂. Increased production of gene transcripts involved in photosynthesis is correlated to increased photosynthetic rates, as seen in physiological

studies of clone 216 (Noormets *et al.*, 2001a,b; Sharma *et al.*, 2004). Another EST showing higher expression under elevated CO₂ was xyloglucan endotransglycosylase, which is responsible for the wall-loosening required for plant cell expansion (Fry *et al.*, 1992). This expression is correlated with the up-regulation of genes coding for various elements of the cytoskeleton associated with growth, such as the alpha and beta subunits of tubulin, and several actin-depolymerizing factors. The expression of these genes may contribute to larger leaf size under elevated CO₂ exposure, as seen in many physiological studies (Ferris *et al.*, 2001). Genes encoding auxin-binding proteins were up-regulated under long-term elevated CO₂. The auxin-binding protein is known to mediate cell expansion and possibly the cell cycle involved in plant growth (Timpte, 2001). In many physiological studies, stimulation of growth (Tjoelker *et al.*, 1998b; Isebrands *et al.*, 2001) and increased cell expansion (Taylor *et al.*, 2003) have been reported with elevated CO₂ exposure. Increased growth under elevated CO₂ could be a result, in part, of triggering of the auxin-binding gene. Genes such as *ABP* were consistently found to be up-regulated over the years of this study under elevated CO₂. However, it is not possible to determine whether differences were attributable to cross-hybridization between different auxin binding protein (ABP) mRNA species or to different regulation profiles within this large gene family.

Down-regulated genes under elevated CO₂ included drought-induced aquaporin plasma membrane intrinsic protein PIPa2, which is responsible for water movement across the membranes. The reduced transcript abundances of these ESTs suggest that plants may be able to manage water efficiently in an elevated CO₂ environment. Tjoelker *et al.* (1998a) reported that plants had better water management under elevated CO₂, as carbon fixed per unit area in the leaf was greater than the water loss, contributing to increased water-use efficiency.

Expression of the phenylpropanoid pathway gene flavanone 3-beta-hydroxylase, which is involved in flavanoid metabolism, was also significantly reduced. Flavanoids consist of various secondary metabolites such as anthocyanins, which give color to flowers and leaves and are regulated by environmental factors or expressed as defense responses to stresses (Winkel-Shirley, 2003). Other genes coding for various enzymes of the secondary metabolism were down-regulated in the elevated CO₂ treatment, such as chalcone-flavonone isomerase and cinnamate-4-hydroxylase. These genes also showed similar patterns of expression in all the treatments and were up-regulated under elevated O₃ and combined elevated CO₂ and O₃. Another secondary metabolism gene, naringenin-chalcone synthase, was overexpressed in these treatments but not under elevated CO₂ alone. Rubisco activase, which controls the overall activity of photosynthesis by regulating enzyme rubisco, showed reduced expression under elevated CO₂. This may suggest that, under elevated CO₂ treatment, the photosynthetic rate can become limiting because of a lack of availability of the rubisco enzyme to fix CO₂, as CO₂ levels

become saturated inside the leaf tissue in an elevated CO₂ environment (Noormets *et al.*, 2001a).

The application of pattern-specific SOM clustering allowed us to obtain lists of genes that are treatment specific. This method of calculation finds genes with similar expression patterns (without regard to the magnitude of ratios), where up- and down-regulation ratios are determined between 1 and 0. Using this method, the cDNA array global expression profiles can be streamlined and highly defined and reduced to a few critical genes that are responsible for treatment-specific responses. These genes would serve as good markers for future studies to elucidate the genotypic differences among aspen clones with differing tolerance to elevated CO₂ and O₃, alone and in combination. Validation by RNA blots confirmed the utility of this approach to identify critical genes responsive to specific treatment effects.

Under combined elevated CO₂ and O₃, there have been reports of amelioration of the negative O₃ response in aspen (Volin & Reich, 1996). However, our results with clone 216 showed that CO₂ in some cases exacerbated the responses observed under elevated O₃, as shown by gene expression profiles in clusters c3, c10 and c13 (Fig. 2). In this study, the expression of some genes under the combined treatment did not show a reversal of the effects of O₃ compared with the individual gases. At elevated O₃, genes associated with defense and secondary metabolism pathways were up-regulated under the combined treatment and a number of photosynthesis genes were down-regulated, indicating that elevated CO₂ was not able to ameliorate all the effects of elevated O₃. While the clustering analysis showed that there were no specific profiles related to the combined treatment of CO₂ and O₃, it is also interesting to note that, under the combined treatment of CO₂ and O₃, more genes were up-regulated than in the treatment with O₃ or CO₂ alone.

Conclusions

Our results suggest that 5-yr-old aspen trees exposed to elevated CO₂ and O₃, alone and in combination, continue to show substantial differences in gene expression patterns. These patterns fit the overall patterns of aspen physiology and growth as summarized by Karnosky *et al.* (2003). It was also evident that elevated CO₂ was not able to ameliorate all the negative effects of O₃. We also found in our study that the interacting effects of elevated CO₂ and O₃ resulted in regulation of expression of genes that were not seen with the individual gas treatments. Further analyses are required on several aspen clones with varying degrees of ozone tolerance to determine how the other genotypes of aspen respond to both elevated O₃ and CO₂, and to determine which genotypes are best suited to adapt to the changing global conditions of elevated CO₂ and O₃.

In this environmental genomic study, we also observed expression profiles for some genes that showed different transcript abundances in RNA sampled in the years 2001 and 2002.

These contrasting profiles highlight the importance of the contribution of other abiotic and/or biotic stresses to gene regulation, in addition to the treatments applied in the FACE project. However, the strong correlation with physiological observations also emphasizes that global gene expression analysis is possible with samples from trees growing in field conditions. This approach lays the foundations for future studies on ecological genomics.

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Supplementary material

The following material is available as supplementary material at <http://www.blackwellpublishing.com/products/journals/suppmat/NPH/NPH1422/NPH1422sm.htm>

Table S1 List of the 238 significantly regulated ESTs analyzed in our expression analysis

Table S2 Gene expression data for CO₂ treatment

Table S3 Gene expression data for O₃ treatment

Table S4 Gene expression data for CO₂ + O₃ treatment

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