Gene expression patterns of trembling aspen trees following long-term exposure to interacting elevated CO_2 and tropospheric O_3

P. Gupta[1]*, S. Duplessis[2]*, H. White[3], D. F. Karnosky[1], F. Martin[2] and G. K. Podila[3]

[1]School of Forest Resources and Environmental Science, Michigan Technological University, 1400 Townsend Drive, Houghton, MI 49931, USA; [2]UMR 1136 INRA/UHP–Nancy, 'Interactions Arbres/Micro-organismes', Centre INRA de Nancy, 54280 Champenoux, France; [3]Department of Biological Sciences, University of Alabama, Huntsville, AL 35899, USA; *These authors contributed equally to this work

Author for correspondence:
Gopi K. Podila
Tel: +1 256 824 6263
Fax: +1 256 824 6305
Email: podilag@uah.edu

Received: *14 February 2005*
Accepted: *16 February 2005*

Summary

• Expression of 4600 poplar expressed sequence tags (ESTs) was studied over the 2001–2002 growing seasons using trees of the moderately ozone (O_3)-tolerant trembling aspen (*Populus tremuloides*) clone 216 exposed to elevated CO_2 and/or O_3 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 CO_2 treatment, relatively small numbers of genes were up-regulated, whereas in the O_3 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 O_3, with or without CO_2 treatment. Interestingly, the combined treatment of CO_2 plus O_3 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 CO_2 and O_3 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.

New Phytologist (2005) **167**: 129–142

© *New Phytologist* (2005) **doi**: 10.1111/j.1469-8137.2005.01422.x

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 (CO_2) (Keeling *et al.*, 1995) and ozone (O_3) (Fowler *et al.*, 1999; Ryerson *et al.*, 2001) are increasing rapidly. Elevated atmospheric CO_2 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 CO_2 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 CO_2 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 CO_2 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 CO_2 on antioxidant transcripts has also been reported (Schwanz & Polle, 1998; Sehmer *et al.*, 1998).

Tropospheric O_3 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 ($O_2 \cdot -$) and hydrogen peroxide (H_2O_2). 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 O_3 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 O_3 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 O_3. 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 O_3 will co-occur in the atmosphere with elevated atmospheric CO_2 over large areas of the world's forests. Many studies have shown that elevated CO_2 reduces the adverse effects of elevated O_3 (Volin & Reich, 1996; Booker *et al.*, 1997; Grams *et al.*, 1999), while other studies have shown that elevated CO_2 exacerbates the negative effects of elevated O_3 (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 CO_2 did not ameliorate the harmful effects of O_3. 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 O_3 and elevated atmospheric CO_2, 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 CO_2 and O_3, alone and in combination, for the past 5 yr (Karnosky *et al.*, 2003). For this study, we examined aspen clone 216, which is moderately O_3-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 CO_2 or elevated O_3 alone. In our study, we collected samples from trees that had grown under elevated CO_2 and O_3 over their entire 5-yr life histories.

The objective of this study was to examine the effects of elevated CO_2 and/or O_3 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 CO_2 and O_3, 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; CO_2 concentration 360 ppm), elevated CO_2 (560 ppm), elevated O_3 (1.5 times ambient) and elevated CO_2 plus elevated O_3 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 O_3-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 O_3-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 *Bio*Grid arrayer (*Bio*Robotics, 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 (O_3/control; CO_2/control; combined CO_2 + O_3/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 (CO_2 vs control, Table S2; O_3 vs control, Table S3; $CO_2 + O_3$ 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 (CO_2 conditions), where the PPDE calculation was impossible because of the data distribution in CO_2 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 O_3 and elevated atmospheric CO_2, 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 CO_2 and O_3, 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 O_3-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 CO_2, elevated O_3 and combined elevated CO_2 and O_3).

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 CO_2 and O_3, alone or in combination, we performed a large-scale expression analysis using a nylon cDNA array. Radioactive [^{33}P] cDNA probes were prepared from replicate sets (years 2001 and 2002) of RNA extracted from aspen leaves exposed to elevated concentrations of CO_2 and O_3, as shown in Table 1. Radiolabeled probes were incubated with 4608-element nylon

Table 1 Summary of treatment exposures for the five years in which aspen (*Populus tremuloides*) trees were exposed to elevated CO_2 and O_3, alone and in combination, at the Aspen FACE facility

	Year				
	1998	1999	2000	2001	2002
Pollutant					
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 (+ O3 and + CO_2 + O_3)	97.8	89	79.9	90.7	80.8
Seasonal exposure duration (d)	166	143	139	143	138

[1]Daytime average (ppm).
[2]Daytime mean concentration (ppb).
[3]Sum 0 (ppmh).

cDNA arrays. These cDNAs correspond to *Populus trichocarpa* Torr. and Gray × *P. deltoides* Bartr. 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 CO_2 and O_3, alone or in combination, were lower, from 0.67 (elevated O_3) to 0.88 (combined elevated CO_2 and O_3), 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 CO_2 treatment between year-replicates, 167 in the elevated O_3 treatment and 111 in the combined elevated CO_2 and O_3 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 CO_2 treatment; 95 (88 up- and seven down-regulated) in the case of the elevated O_3 treatment and 51 (44 up- and seven down-regulated) in the case of the combined elevated CO_2 and O_3 treatment. The relatively small number of genes showing a significant regulation profile between 2001 and 2002 in the elevated CO_2 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 CO_2 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 O_3 and combined CO_2 and O_3 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 (O_3 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

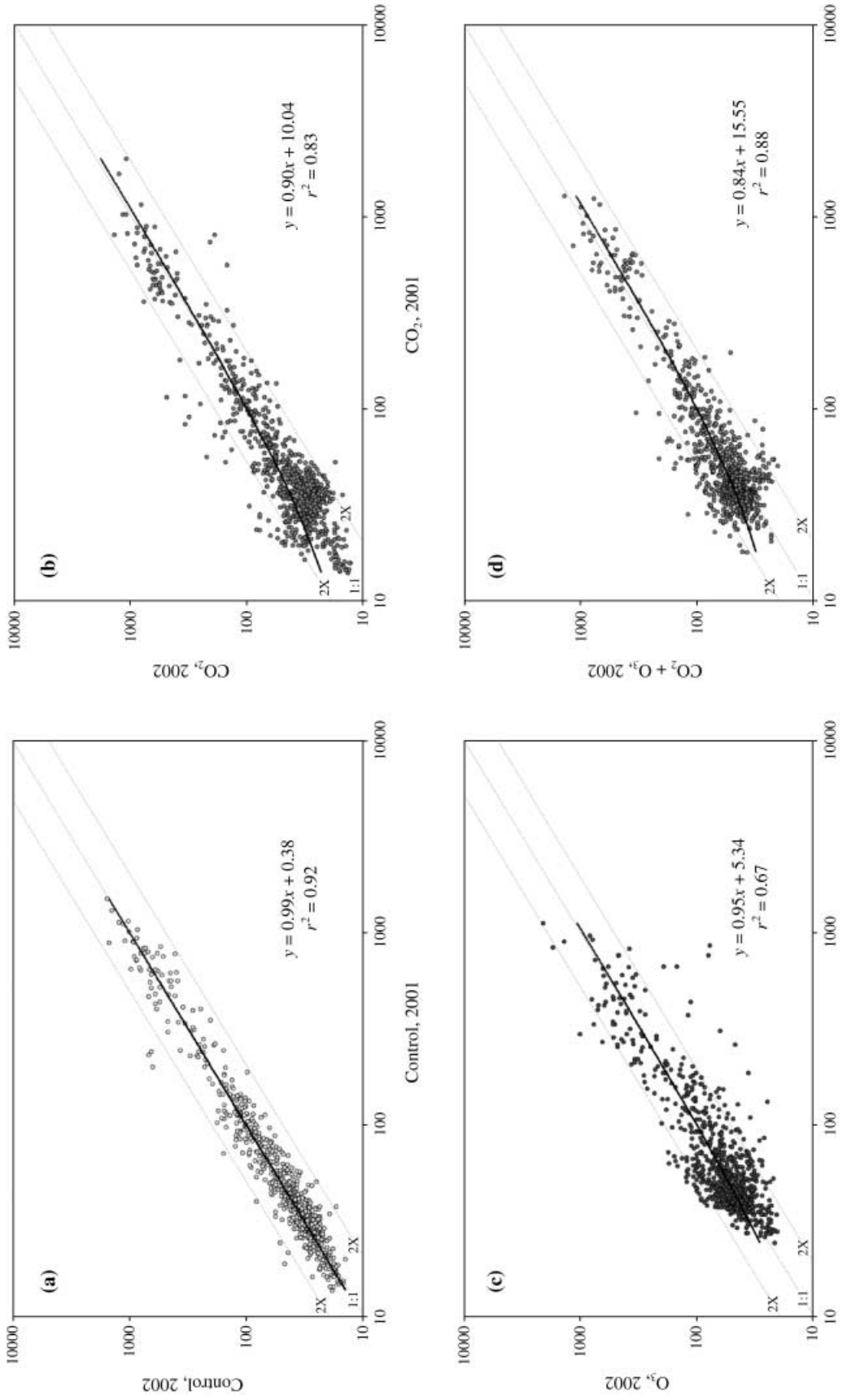

Fig. 1 Scatter plots comparing the signal intensities of pairs of arrays hybridized with two sets of cDNA probes prepared from RNA extracts from two year-replicates (2001 on the *x*-axis and 2002 on the *y*-axis). (a) Normalized mRNA abundance of 801 expressed sequence tags (ESTs) measured in leaves of aspen (*Populus tremuloides*) grown in control conditions. (b) Normalized mRNA abundance of 801 ESTs measured in leaves of aspen grown under elevated CO_2 concentration. (c) Normalized mRNA abundance of 801 ESTs measured in leaves of aspen grown under elevated O_3 concentration. (d) Normalized mRNA abundance of 801 ESTs measured in leaves of aspen grown under combined elevated CO_2 and O_3 concentrations. All axes show a $\log_{10}$ 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 CO_2, elevated O_3 and combined elevated CO_2 and O_3 concentrations compared with control conditions (C)

Clone ID	Accession no.	Gene function	CO_2/C	ln *P*-value	O_3/C	ln *P*-value	O_3 + CO_2/C	ln *P*-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. CO_2).

Cluster analysis of gene expression

We grouped the expression profiles of the 238 significantly regulated ESTs in the elevated CO_2 and/or O_3 treatments (alone or in combination) with the program GENECLUSTER

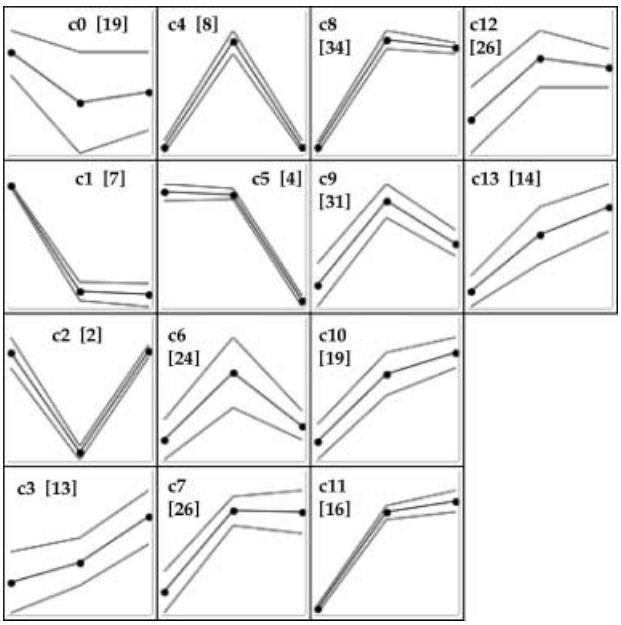

Fig. 2 Self-organizing map (SOM) clusters of expression profiles measured in leaves of aspen (*Populus tremuloides*) grown under elevated CO_2, elevated O_3 and combined elevated CO_2 and O_3 concentrations for genes that showed significant regulation of gene expression in at least one treatment when 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 CO_2 treatment, most of the clusters corresponded to genes more highly expressed under elevated O_3 alone (cluster (c) 4, c6 and c9) or in combination with CO_2 (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 CO_2, alone or in combination with O_3, when compared with the treatment with elevated O_3 alone. This last point underlines the substantial impact of elevated O_3 alone, even on O_3-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 O_3, alone or combined with CO_2, than in elevated CO_2 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 CO_2 treatment, while a much larger number of genes were up- or down-regulated in the O_3 treatment, alone or in combination with CO_2. In the CO_2 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 O_3 treatment were similarly altered in the combined treatment with CO_2. 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 O_3, a number of ESTs that are involved in signal transduction,

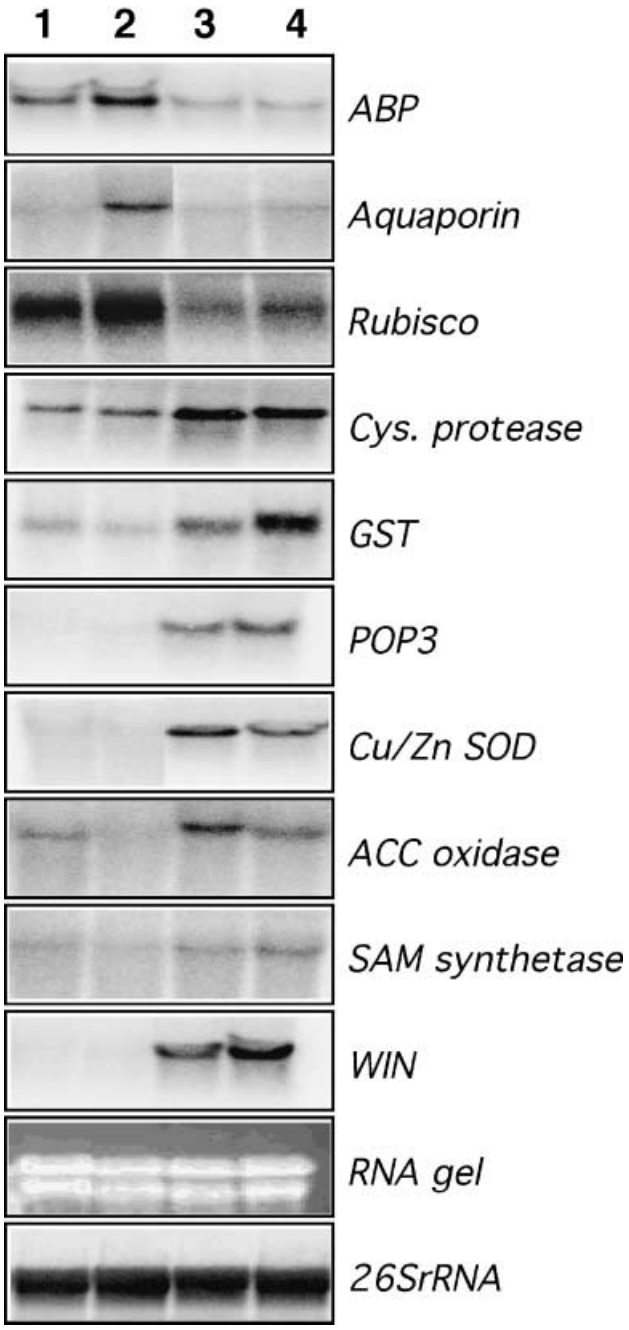

Fig. 3 RNA blot analyses for validation of cDNA arrays. Hybridization of 10 poplar cDNA probes to total RNAs extracted from aspen (*Populus tremuloides*) grown in control (lane 1), elevated CO_2 concentration (lane 2), elevated O_3 concentration (lane 3) and combined elevated CO_2 and O_3 concentration (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 ^{32}P-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 bisphosphate 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 O_3 environments, high concentrations of ethylene are produced (Wolfgang *et al*., 2002). Senescence-associated genes (SAGs) that were highly expressed in elevated O_3 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 O_3. A thaumatin-like protein-encoding gene was also up-regulated under elevated O_3. 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 O_3 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 O_3, including genes coding for rubisco activase, the small subunit of ribulose bisphosphate 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 O_3 may contribute to the reduced photosynthetic rates observed under elevated O_3 (Noormets *et al*., 2001a,b). In addition, we detected evidence of premature senescence being triggered under elevated O_3 (Sharma *et al*., 2003).

It is well known that O_3 causes premature foliar senescence and early leaf abscission (Landry & Pell, 1993; Volin & Reich, 1996; Nali *et al*., 1998). Genes up-regulated under elevated O_3 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 O_3 exposure (Tamaoki *et al*., 2003), such as plant defense genes (mainly up-regulated) and energy-related genes (down-regulated).

Under elevated CO_2, 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 CO_2. 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 CO_2 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 CO_2 exposure, as seen in many physiological studies (Ferris *et al.*, 2001). Genes encoding auxin-binding proteins were up-regulated under long-term elevated CO_2. 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 CO_2 exposure. Increased growth under elevated CO_2 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 CO_2. 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 CO_2 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 CO_2 environment. Tjoelker *et al.* (1998a) reported that plants had better water management under elevated CO_2, 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 CO_2 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 O_3 and combined elevated CO_2 and O_3. Another secondary metabolism gene, naringenin-chalcone synthase, was overexpressed in these treatments but not under elevated CO_2 alone. Rubisco activase, which controls the overall activity of photosynthesis by regulating enzyme rubisco, showed reduced expression under elevated CO_2. This may suggest that, under elevated CO_2 treatment, the photosynthetic rate can become limiting because of a lack of availability of the rubisco enzyme to fix CO_2, as CO_2 levels become saturated inside the leaf tissue in an elevated CO_2 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 CO_2 and O_3, 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 CO_2 and O_3, there have been reports of amelioration of the negative O_3 response in aspen (Volin & Reich, 1996). However, our results with clone 216 showed that CO_2 in some cases exacerbated the responses observed under elevated O_3, 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 O_3 compared with the individual gases. At elevated O_3, 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 CO_2 was not able to ameliorate all the effects of elevated O_3. While the clustering analysis showed that there were no specific profiles related to the combined treatment of CO_2 and O_3, it is also interesting to note that, under the combined treatment of CO_2 and O_3, more genes were up-regulated than in the treatment with O_3 or CO_2 alone.

Conclusions

Our results suggest that 5-yr-old aspen trees exposed to elevated CO_2 and O_3, 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 CO_2 was not able to ameliorate all the negative effects of O_3. We also found in our study that the interacting effects of elevated CO_2 and O_3 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 O_3 and CO_2, and to determine which genotypes are best suited to adapt to the changing global conditions of elevated CO_2 and O_3.

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.

Acknowledgements

We thank Dr A. Kohler for providing the poplar 4608-element nylon cDNA arrays and for valuable discussions during the writing of the manuscript. We also thank the two anonymous referees for their comments and suggestions on the manuscript. Part of this work was sponsored by the Office of Science (BER), U.S. Department of Energy Grant No. DEFG02-04ER63792, to DFK and GKP. The research utilized, in part, the DNA Sequencing and Functional Genomics Facilities at INRA-Nancy financed by the INRA and Région de Lorraine through the Institut Fédérateur de Recherche no. 110.

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 CO_2 treatment

Table S3 Gene expression data for O_3 treatment

Table S4 Gene expression data for CO_2 + O_3 treatment

References

Akkapeddi A, Noormets A, Deo BK, Karnosky DF, Podila GK. 1999. Gene structure and expression of aspen cytosolic Cu, Zn-superoxide dismutase (PtSodCc1). *Plant Science* **143**: 151–162.

Baldi P, Hatfield GW. 2002. *DNA microarrays and gene expression from experiment to data analysis and modeling*. Cambridge, UK: Cambridge University Press.

Bernhard WR, Matile P. 1994. Differential expression of glutamine synthetase genes during the senescence of *Arabidopsis thaliana* rosette leaves. *Plant Science* **98**: 714.

Bobbink R. 1998. Impacts of tropospheric ozone and airborne nitrogenous pollutants on natural and seminatural ecosystems: a commentary. *New Phytologist* **139**: 11–23.

Booker FL, Reid CD, Brunschon-Harti S, Ficus EL. 1997. Photosynthesis and photorespiration in soybean (*Glycine max* (L.) Merr) chronically exposed to elevated carbon dioxide and ozone. *Journal of Experimental Botany* **48**: 1843–1852.

Brazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, Stoeckert C, Aash J, Ansorge W, Ball CA, Causton HC, Gaasterland T, glenisson P, Holstege FCP, Kim IF, Markowitz V, Matese JC, Parkinson H, robinson A, Sarkans U, Schulze-Kremer S, Stewart J, Taylor R, Vilo J, Vingron M. 2001. Minimum information about a microarray experiment (MIAME) – toward standards for microarray data. *Nature Genetics* **29**: 365–371.

Broadmeadow M. 1998. Ozone and forest trees. *New Phytologist* **139**: 123–125.

Bryant J, Taylor G, Frehner M. 1998. Photosynthetic acclimation to elevated CO_2 is modified by source:sink balance in three component species of chalk grassland swards grown in a free air carbon dioxide enrichment (FACE) experiment. *Plant, Cell & Environment* **21**: 159–168.

Chang S, Puryear J, Cairney J. 1993. A simple method for isolating RNA from pine trees. *Plant Molecular Biology Reporter* **11**: 113–116.

Cheong YH, Chang HS, Gupta R, Wang X, Zhu T, Luan S. 2002. Transcriptional profiling reveals novel interactions between wounding, pathogen abiotic stress and hormonal responses in Arabidopsis. *Plant Physiology* **129**: 661–677.

Conklin PL, Last RL. 1995. Differential accumulation of antioxidant mRNAs in *Arabidopsis thaliana* exposed to ozone. *Plant Physiology* **109**: 203–212.

Dickson RE, Lewin KF, Isebrands JG, Coleman MD, Heilman WE, Riemenschneider DE, Sober J, Host GE, Zak DR, Hendrey GR, Pregitzer KS, Karnosky DF. 2000. *Forest atmosphere carbon transfer and storage (FACTS-II). The Aspen Free-air CO_2 and O_3 Enrichment (FACE) Project: an overview*. General technical report NC-214. St Paul, MN, USA: USDA Forest Service North Central Research Station, 68.

Drake BG, Gonzalez-Meler MA, Long SP. 1997. More efficient plants: a consequence of rising atmospheric CO_2. *Annual Review of Plant Physiology and Plant Molecular Biology* **48**: 609–639.

Duplessis S, Courty PE, Tagu D, Martin F. 2005. Transcript patterns associated with ectomycorrhiza development in *Eucalyptus globulus* and *Pisolithus microcarpus*. *New Phytologist* **165**: 599–611.

Ederli L, Pasqualini S, Batini P, Antonielli M. 1997. Photoinhibition and oxidative stress: effects on xanthophyll cycle, scavenger enzymes and abscisic acid content in tobacco plants. *Journal of Plant Physiology* **151**: 422–428.

Eisen MB, Spellman PT, Brown PO, Botstein D. 1998. Cluster analysis and display of genome-wide expression patterns. *Proceedings of the National Academy of Sciences, USA* **95**: 14863–14768.

Ferris R, Sabatti M, Miglietta F, Mills RF, Taylor G. 2001. Leaf area is stimulated in *Populus* by free air CO_2 enrichment (POPFACE), through increased cell expansion and production. *Plant, Cell & Environment* **24**: 305–315.

Fowler D, Cape JN, Coyle M, Flechard C, Kuylenstierna J, Hicks K, Derwent D, Johnson C, Stevenson D. 1999. The global exposure of forests to air pollutants. *Water, Air and Soil Pollution* **116**: 5–32.

Fry SC, Smith RC, Renwick KF, Martin DJ, Hodge SK, Matthews KJ. 1992. Xyloglucan endotransglycosylase, a new wall-loosening enzyme activity from plants. *Biochemistry Journal* **282**: 821–828.

Gesch RW, Boote KJ, Vu JCV, Allen LH Jr, Bowes G. 1998. Changes in growth CO_2 result in rapid adjustments of ribulose-1, 5-bisphosphate carboxylase/oxygenase small subunit gene expression in expanding and mature leaves of rice. *Plant Physiology* **118**: 521–529.

Glick RE, Schlagnhaufer CD, Arteca RN, Pell EJ. 1995. Ozone-induced ethylene emission accelerates the loss of ribulose1, 5-bisphosphate carboxylase/oxygenase and nuclear-encoded mRNAs in senescing potato leaves. *Plant Physiology* **109**: 891–898.

Grams TEE, Anegg S, Haberle KH, Langebartels C, Matyssek R. 1999. Interactions of chronic exposure to elevated CO_2 and O_3 levels in the photosynthetic light and dark reactions of European beech (*Fagus sylvatica*). *New Phytologist* **144**: 95–107.

Isebrands JG, McDonald EP, Kruger E, Hendrey G, Percy K, Pregitzer K, Sober J, Karnosky DF. 2001. Growth responses of *Populus tremuloides* clones to interacting elevated carbon dioxide and tropospheric ozone. *Environmental Pollution* **115**: 359–371.

Jones AM, Herman EM. 1993. KDEL-containing auxin-binding protein is secreted to the plasma membrane and cell wall. *Plant Physiology* **101**: 595–606.

Karnosky DF, Mankovska B, Percy K, Dickson RE, Podila GK, Sober J, Noormets A, Hendrey G, Coleman MD, Kubiske M, Pregitzer KS, Isebrands JG. 1999. Effects of tropospheric O_3 on trembling aspen and interactions with CO_2 results from an O_3 gradient and a FACE experiment. *Water, Air and Soil Pollution* **116**: 311–322.

Karnosky DF, Percy KE, Xiang B, Callan B, Noormets A, Mankovska B, Hopkin A, Sober J, Jones W, Dickson RE, Isebrands JG. 2002. Interacting elevated CO_2 and tropospheric O_3 predisposes aspen (*Populus tremuloides* Michx.) to infection by rust (*Melampsora medusae* f.sp. *tremuloidae*). *Global Change Biology* **8**: 329–338.

Karnosky DF, Podila GK, Gagnon Z, Pechter P, Akkapeddi A, Coleman M, Dickson RE, Isebrands JG. 1998. Genetic control of responses to interacting O_3 and CO_2 in *Populus tremuloides*. *Chemosphere* **36**: 807–812.

Karnosky DF, Zak DR, Pregitzer KS, Awmack CS, Bockheim JG, Dickson RE, Hendrey GR, Host GE, King JS, Kopper BJ, Kruger EL, Kubiske ME, Lindroth RL, Mattson WJ, McDonald EP, Noormets A, Oksanen E, Parsons WFJ, Percy KE, Podila GK, Riemenschneider DE, Sharma P, Thakur R, Sober A, Sober J, Jones WS, Anttonen S, Vapaavuori E, Mankovska B, Heilman W, Isebrands JG. 2003. Low levels of tropospheric O_3 moderate responses of temperate hardwood forests to elevated CO_2: a synthesis of results from the Aspen FACE project. *Functional Ecology* **17**: 289–304.

Keeling CD, Whort TP, Wahlen M, Vander Plicht J. 1995. Interannual extremes in the rate of rise of atmospheric carbon dioxide since 1980. *Nature* **375**: 666–670.

King JS, Pregitzer KS, Zak DR, Sôber J, Isebrands JG, Dickson RE, Hendrey GR, Karnosky DF. 2001. Fine root biomass and fluxes of soil carbon in young stands of paper birch and trembling aspen as affected by elevated atmospheric CO_2 and tropospheric O_3. *Oecologia* **128**: 237–250.

Kohler A, Delaruelle C, Martin D, Encelot N, Martin F. 2003. The poplar root transcriptome: analysis of 7000 expressed sequence tags. *FEBS Letters* **542**: 37–41.

Kull O, Sober A, Coleman MD, Dickson RE, Isebrands JG, Gagnon Z, Karnosky DF. 1996. Photosynthetic responses of aspen clones to simultaneous exposures of ozone and CO_2. *Canadian Journal of Forest Research* **26**: 639–648.

Landry LG, Pell EJ. 1993. Modification of rubisco and altered proteolytic activity in O_3-stressed hybrid poplar (*Populus maximowizii* × *trichocarpa*). *Plant Physiology* **101**: 1355–1362.

Larson PR, Isebrands JG. 1971. The plastochron index as applied to developmental studies of cottonwood. *Canadian Journal of Forest Research* **1**: 1–11.

Lehrach H, D.Diamond JM, Wozney, H. Boedtker. **1977.** RNA molecular weight determination by gel electrophoresis under denaturing conditions: a critical reexamination. *Biochemistry* **16**: 4743–4751.

Lindroth RL, Kinney KK, Platz CL. 1993. Responses of deciduous trees to elevated atmospheric CO_2: productivity, phytochemistry and insect performance. *Ecology* **74**: 763–777.

Lindroth RL, Roth S, Kruger EL, Volin JC, Koss PA. 1997. CO_2-mediated changes in aspen chemistry: effects on gypsy moth performance and susceptibility to virus. *Global Change Biology* **3**: 279–289.

Lohman KN, Gan S, John MC, Amasino RM. 1994. Molecular analysis of natural senescence in *Arabidopsis thaliana*. *Physiologia Plantarum* **92**: 322–328.

Long AD, Mangalam HJ, Chan BYP, Tolleri L, Hatfield GW, Baldi P. 2001. Improved statistical inference from DNA microarray data using analysis of variance and a bayesian statistical framework. *Journal of Biological Chemistry* **276**: 19937–19944.

Loreto F, Velikova V. 2001. Isoprene produced by leaves protects the photosynthetic apparatus against ozone damage, quenches ozone products, and reduces lipid peroxidation of cellular membranes. *Plant Physiology* **127**: 1781–1787.

Lutz C, Anegg S, Gerant D, Slaoui-Sosse B, Gerard J, Dizengremel P. 2000. Beech trees exposed to high CO_2 and to simulated summer ozone levels: effects on photosynthesis, chloroplast components and leaf enzyme activity. *Physiologia Plantarum* **109**: 252–259.

McDonald EP, Kruger EL, Riemenschneider DE, Isebrands JG. 2002. Competitive status influences tree growth responses to elevated CO_2 and O_3 in aggrading aspen stands. *Functional Ecology* **16**: 792–801.

Miller JD, Richard NA, Pell EJ. 1999. Senescence-associated gene expression during ozone-induced leaf senescence in arabidopsis. *Plant Physiology* **120**: 1015–1024.

Moore BD, Cheng SH, Sims D, Seeman JR. 1999. The biochemical and molecular basis for photosynthetic acclimation of elevated atmospheric CO_2. *Plant, Cell & Environment* **22**: 567–582.

Nali C, Guidi L, Filippi F, Soldatini GF, Lorenzini G. 1998. Photosynthesis of two poplar clones contrasting in O_3 sensitivity. *Trees* **12**: 196–200.

Nie GY, Tomasevic M, Baker NR. 1993. Effects of ozone on the photosynthetic apparatus and leaf proteins during leaf development in wheat. *Plant, Cell & Environment* **16**: 643–651.

Noormets A, McDonald EP, Dickson RE, Kruger EL, Sôber A, Isebrands JG, Karnosky DF. 2001a. The effect of elevated CO_2 and O_3 on leaf and branch level photosynthesis and potential plant level carbon gain in aspen. *Trees* **15**: 262–270.

Noormets A, Sôber A, Pell EJ, Dickson RE, Podila GK, Sôber J, Isebrands JG, Karnosky DF. 2001b. Stomatal and non-stomatal limitation to photosynthesis in two trembling aspen (*Populus tremuloides* Michx.) clones exposed to elevated CO_2 and/or O_3. *Plant, Cell & Environment* **24**: 327–336.

Norby RJ, Wullschleger SD, Gunderson CA, Johnson DW, Ceulemans R. 1999. Tree responses to rising CO_2 in field experiments: implications for the future forest. *Plant, Cell & Environment* **22**: 683–714.

Oren R, Ellsworth DE, Johnsen KH, Phillips N, Ewers BE, Maier C, Schäfer KVR, McCarthy H, Hendrey G, McNulty SG, Katul GG. 2001. Soil fertility limits carbon sequestration by forest ecosystems in a CO_2-enriched atmosphere. *Nature* **411**: 469–472.

Pell EJ, Schlagnhaufer CD, Arteca RN. 1997. Ozone-induced oxidative stress: mechanisms of action and reaction. *Physiologia Plantarum* **100**: 264–273.

Percy KE, Awmack CS, Lindroth RL, Kubiske ME, Kopper BJ, Isebrands JG, Pregitzer KS, Hendrey GR, Dickson RE, Zak DR, Oksanen E, Sober J, Harrington R, Karnosky DF. 2002. Altered performance of forest pests under atmospheres enriched by CO_2 and O_3. *Nature* **6914**: 403–407.

Peter M, Courty P-E, Kohler A, Delaruelle C, Martin D, Tagu D, Frey-klett P, Duplessis S, Chalot M, Podila G, Martin F. 2003. Analysis of expressed sequence tags from the ectomycorrhizal basidiomycetes *Laccaria bicolor* and *Pisolithus microcarpus*. *New Phytologist* **159**: 117–129.

Polle A, Pell E. 1999. Role of carbon dioxide in modifying the plant response to ozone. In: Luo Y, Mooney HA, eds. *Carbon dioxide and environmental stress*. New York, USA: Academic Press, 193–213.

Rao MV, Davis KR. 1999. Ozone-induced cell death occurs via two distinct mechanisms in Arabidopsis: the role of salicylic acid. *The Plant Journal of Cell and Molecular Biology* **17**: 603–614.

Rao MV, Lee HI, Creelman RA, Mullet JE, Davis KR. 2000. Jasmonic acid signaling modulates ozone-induced hypersensitive cell death. *The Plant Cell* **12**: 1633–1646.

Rey A, Jarvis PG. 1998. Long-term photosynthetic acclimation to increased atmospheric CO_2 concentration in young birch (*Betula pendula*) trees. *Tree Physiology* **18**: 441–450.

Reymond P, Weber H, Damond M, Farmer EE. 2000. Differential gene expression in response to mechanical wounding and insect feeding in Arabidopsis. *Plant Cell* **12**: 707–719.

Ryerson TB, Trainer M, Holloway JS, Parrish DD, Huey LG, Sueper DT, Frost GJ, Donnelly SG, Schauffler S, Atlas S, Kuster WC, Goldan PD, Hubler G, Meagher JF, Fehsenfeld FC. 2001. Observations of ozone formation in plant plumes and implications for ozone control strategies. *Science* **292**: 719–723.

Scalet M, Federico R, Guido MC, Manes F. 1994. Peroxidase activity and polyamine changes in response to ozone and simulated acid rain in aleppo pine needles. *Environmental and Experimental Botany* **35**: 417–425.

Schwanz P, Catherine P, Vivin P, Dreyer E, Guehl JM, Polle A. 1996. Responses of antioxidant systems to drought stress in pendunculate oak and maritime pine as modulated by elevated CO_2. *Plant Physiology* **110**: 393–402.

Schwanz P, Polle A. 1998. Antioxidative systems, pigment and protein contents in leaves of adult Mediterranean oak species (*Quercus pubescens* and *Q. ilex*) with lifetime exposure to elevated CO_2. *New Phytologist* **140**: 411–423.

Sehmer L, Fontaine V, Antoni F, Dizengremel P. 1998. Effects of ozone and elevated atmospheric carbon dioxide on carbohydrate metabolism of spruce needles. Catabolic and detoxification pathways. *Physiologia Plantarum* **102**: 605–611.

Sharma P, Sober A, Sober J, Podila GK, Kubiske ME, Mattson WJ, Isebrands JG, Karnosky DF. 2003. Moderation of [CO_2]-induced gas exchange responses by elevated tropospheric O_3 in trembling aspen and sugar maple. *Ecologia-Bratislava* **22** (Supplement): 304–317.

Stasolla C, Belmonte MF, van Zyl L, Craig DL, Liu W, Yeung EC, Sederoff RR. 2004. The effect of reduced glutathione on morphology and gene expression of white spruce (*Picea glauca*) somatic embryos. *Journal of Experimental Botany* **55**: 695–709.

Sterky F, Bhalerao RR, Unneberg P, Segerman B, Nilsson P, Brunner AM, Charbonnel-Campaa L, Jonsson Lindvall J, Tandre K, Strauss SH, Sundberg B, Gustafsson P, Uhlén M, Bhalerao RP, Nilsson O, Sandberg G, Karlsson J, Lunderberg J, Jansson S. 2004. A *Populus* EST resource for plant functional genomics. *Proceedings of the National Academy of Sciences, USA* **101**: 13951–13956.

Tamaoki M, Nakajima N, Kubo A, Aono M, Matsuyama T, Saji H. 2003. Transcriptome analysis of O_3-exposed Arabidopsis reveals that multiple signal pathways act mutually antagonistically to induce gene expression. *Plant Molecular Biology* **53**: 443–456.

Tamayo P, Slonim D, Mesirov J, Zhu Q, Kitareewan S, Dmitrovsky E, Lander ES, Golub TR. 1999. Interpreting patterns of gene expression with self-organizing maps: methods and application to hematopoietic differentiation. *Proceedings of the National Academy of Sciences, USA* **96**: 2907–2912.

Taylor G, Tricker PJ, Zhang FZ, Alston VJ, Miglietta F, Kuzminsky E. 2003. Spatial and temporal effects of free-air CO_2 enrichment (POPFACE) on leaf growth, cell expansion, and cell production in a closed canopy of poplar. *Plant Physiology* **1**: 177–185.

Timpte C. 2001. Auxin binding protein: curiouser and curiouser. *Trends in Plant Science* **6**: 586–590.

Tissue DT, Griffin KL, Ball JT. 1999. Photosynthetic adjustment in field grown ponderosa pine trees after six years of exposure to elevated CO_2. *Tree Physiology* **19**: 221–228.

Tjoelker MG, Oleksyn J, Reich PB. 1998a. Seedlings of five boreal tree species differ in acclimation of net photosynthesis to elevated CO_2 and temperature. *Tree Physiology* **18**: 715–726.

Tjoelker MG, Oleksyn J, Reich PB. 1998b. Temperature and ontogeny mediate growth response to elevated CO_2 in seedlings of five boreal tree species. *New Phytologist* **140**: 197–210.

Tognini M, Ranieri A, Castagna A, Nali C, Lorenzini G, Soldatini GF. 1997. Ozone-induced alterations in thylakoid protein patterns in pumpkin leaves of different age. *Phyton* **37**: 277–282.

Tokarska-Schlattner M, Fink A, Castillo FJ, Crespi P, Crevecoeur M, Greppin H, Tacchini P. 1997. Effects of ozone on the plasma membrane proteins in *Arabidopsis thaliana* (L.) leaves. *Plant, Cell & Environment* **20**: 1205–1211.

Van Damme EJ, Charels D, Menu-Bouaouiche L, Proost P, Barre A, Rouge P, Peumans WJ. 2002. Biochemical, molecular and structural analysis of multiple thaumatin-like proteins from the elderberry tree (*Sambucus nigra* L.). *Planta* **214**: 853–862.

Volin JC, Reich PB. 1996. Interaction of elevated CO_2 and O_3 growth, photosynthesis and respiration of three perennial species grown in low and high nitrogen. *Physiologia Plantarum* **97**: 674–684.

Wellburn FAM, Wellburn AR. 1996. Variable patterns of antioxidant protection but similar ethene emission differences between ozone-fumigated and control treatments in several ozone-sensitive and ozone-tolerant plant selections. *Plant, Cell & Environment* **19**: 754–760.

Will RE, Ceulemans R. 1997. Effects of elevated CO_2 concentration on photosynthesis, respiration and carbohydrate status of coppice *Populus* hybrids. *Physiologia Plantarum* **100**: 933–939.

Winkel-Shirley B. 2003. Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. *Plant Physiology* **126**: 485–493.

Wolfgang M, Barry CS, Tauriainen AA, Betz C, Tuomainen J, Utriainen M, Grierson D, Sandermann H, Langebartels C, Kangas J. 2002. Ethylene synthesis regulated by biphasic induction of 1-aminocyclopropane-1-carboxylic acid synthase and 1-aminocyclopropane-1-carboxylic acid oxidase genes is required for hydrogen peroxide accumulation and cell death in ozone-exposed tomato. *Plant Physiology* **130**: 1918–1926.

Wustman BA, Oksanen E, Karnosky DF, Sôber J, Isebrands JG, Hendrey GR, Pregitzer KS, Podila GK. 2001. Effects of elevated CO_2 and O_3 on aspen clones varying in O_3 sensitivity: can CO_2 ameliorate the harmful effects of O_3? *Environmental Pollution* **115**: 473–481.

van Zyl L, von Arnold S, Bozhkov P, Chen Y, Egertsdotter U, MacCay J, Sederoff RR, Shen J, Zelena L, Clapham DH. 2002. Heterologous array analysis in Pinaceae: hybridization of *Pinus taeda* cDNA arrays with cDNA from needles and embryogenic cultures of *P. taeda*, *P. sylvestris* or *Picea abies*. *Comparative and Functional Genomics* **3**: 306–318.

About *New Phytologist*

- *New Phytologist* is owned by a non-profit-making **charitable trust** dedicated to the promotion of plant science, facilitating projects from symposia to open access for our Tansley reviews. Complete information is available at **www.newphytologist.org**.
- Regular papers, Letters, Research reviews, Rapid reports and both Modelling/Theory and Methods papers are encouraged. We are committed to rapid processing, from online submission through to publication 'as-ready' via *OnlineEarly* – the 2004 average submission to decision time was just 30 days. Online-only colour is **free**, and essential print colour costs will be met if necessary. We also provide 25 offprints as well as a PDF for each article.
- For online summaries and ToC alerts, go to the website and click on 'Journal online'. You can take out a **personal subscription** to the journal for a fraction of the institutional price. Rates start at £109 in Europe/$202 in the USA & Canada for the online edition (click on 'Subscribe' at the website).
- If you have any questions, do get in touch with Central Office (**newphytol@lancaster.ac.uk**; tel +44 1524 592918) or, for a local contact in North America, the US Office (**newphytol@ornl.gov**; tel +1 865 576 5261).

