

## Molecular targets of elevated [CO<sub>2</sub>] in leaves and stems of *Populus deltoides*: implications for future tree growth and carbon sequestration

Nathalie Druart<sup>A,B</sup>, Marisa Rodríguez-Buey<sup>A</sup>, Greg Barron-Gafford<sup>C</sup>, Andreas Sjödin<sup>A</sup>,  
Rishikesh Bhalerao<sup>B</sup> and Vaughan Hurry<sup>A,D</sup>

<sup>A</sup>Umeå Plant Science Centre, Department of Plant Physiology, Umeå University, S-901 87 Umeå, Sweden.

<sup>B</sup>Umeå Plant Science Centre, Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences, S-901 83 Umeå, Sweden.

<sup>C</sup>Biosphere 2 Laboratory, Columbia University, Oracle AZ 85623, USA. Current address: Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ 85719, USA.

<sup>D</sup>Corresponding author. Email: Vaughan.Hurry@plantphys.umu.se

**Abstract.** We report the first comprehensive analysis of the effects of elevated [CO<sub>2</sub>] on gene expression in source leaf and stem sink tissues in woody plants. We have taken advantage of coppiced *Populus deltoides* (Bartr.) stands grown for 3 years under three different and constant elevated [CO<sub>2</sub>] in the agriforest mesocosms of Biosphere 2. Leaf area per tree was doubled by elevated [CO<sub>2</sub>] but although growth at 800 v. 400 μmol mol<sup>-1</sup> CO<sub>2</sub> resulted in a significant increase in stem biomass, growth was not stimulated at 1200 μmol mol<sup>-1</sup> CO<sub>2</sub>. Growth under elevated [CO<sub>2</sub>] also resulted in significant increases in stem wood density. Analysis of expression data for the 13 490 clones present on POP1 microarrays revealed 95 and 277 [CO<sub>2</sub>]-responsive clones in leaves and stems respectively, with the response being stronger at 1200 μmol mol<sup>-1</sup>. When these [CO<sub>2</sub>]-responsive genes were assigned to functional categories, metabolism-related genes were the most responsive to elevated [CO<sub>2</sub>]. However within this category, expression of genes relating to bioenergetic processes was unchanged in leaves whereas the expression of genes for storage proteins and of those involved in control of wall expansion was enhanced. In contrast to leaves, the genes up-regulated in stems under elevated [CO<sub>2</sub>] were primarily enzymes responsible for lignin formation and polymerisation or ethylene response factors, also known to induce lignin biosynthesis. Concomitant with this enhancement of lignin biosynthesis in stems, there was a pronounced repression of genes related to cell wall formation and cell growth. These changes in gene expression have clear consequences for long-term carbon sequestration, reducing the carbon-fertilisation effect, and the potential for increased lignification may negatively impact on future wood quality for timber and paper production.

**Keywords:** cottonwood, elevated CO<sub>2</sub>, global change, microarray, *Populus*.

### Introduction

The concentration of carbon dioxide in the atmosphere has increased from 280 μmol mol<sup>-1</sup> before the Industrial Revolution to 380 μmol mol<sup>-1</sup> today, and it continues to rise at ~1.8 μmol mol<sup>-1</sup> year<sup>-1</sup> (Field 2001). Associated with this rise in atmospheric [CO<sub>2</sub>], global temperatures are predicted to increase by between 1 and 6°C by the year 2100 (Hansen *et al.* 1999). It is now widely recognised that the terrestrial biosphere is a significant carbon sink that is responding to global climate change (e.g. Myneni *et al.* 2001). Any future control measures implemented to moderate the

effects of increasing greenhouse gases must incorporate this important capacity of the terrestrial ecosystems to sequester carbon, and although we should not exaggerate the capacity of the terrestrial forest sinks to mitigate anthropogenic CO<sub>2</sub> emissions (Falkowski *et al.* 2000), experimental evaluation of molecular- through ecosystem-level responses underpinning feedbacks in the biosphere remains a matter of high priority (Osmond *et al.* 2004).

There is now a wealth of data showing that plants from a wide range of biomes show increased growth and water-use efficiency when exposed to elevated [CO<sub>2</sub>]

(Poorter 1993; Rogers and Dahlman 1993; DeLucia *et al.* 1999; Oren *et al.* 2001). However, these growth enhancements are often not sustained beyond the first couple of years following CO<sub>2</sub> enrichment, due to co-limitation by soil fertility (Conroy *et al.* 1990b; Julkunen-Tiitto *et al.* 1993; Oren *et al.* 2001) or to feedback limitation from sinks, leading to carbon accumulation in the source tissues (Paul and Foyer 2001; Woodward 2002). Related to this apparent sink limitation, the initial stimulation of photosynthesis is frequently followed by downward acclimation (Clough *et al.* 1981; Griffin *et al.* 2000; Jach and Ceulemans 2000). Furthermore, plants from different functional groups tend to respond differently to elevated [CO<sub>2</sub>], with fast-growing C<sub>3</sub> herbaceous species generally responding more strongly than their slower-growing counterparts and woody species showing an intermediate response (Poorter and Navas 2003).

These data leave us with several perplexing questions, not least of which is why such a diversity of plant species show apparent sink limitations in response to elevated [CO<sub>2</sub>] (Paul and Foyer 2001; Woodward 2002; Poorter and Navas 2003). These questions are particularly important if we are to understand how trees and forests, such as the boreal forest that annually stores ~0.7 gigatonnes of carbon per year (Myneni *et al.* 2001), will respond to elevated [CO<sub>2</sub>] in the long term. For example, recent data indicating a significant increase in fine root production and soil respiration under sustained elevated [CO<sub>2</sub>] (Norby *et al.* 2004; Barron-Gafford *et al.* 2005; Trueman and Gonzalez-Meler 2005), suggest possible long-term changes in relative sink strengths. Detailed meta-analyses such as those carried out by Poorter and Navas (2003) clearly point to different biomes, with their unique species mixes, responding differently over the long-term to elevated [CO<sub>2</sub>], resulting in unpredictable consequences for carbon sequestration. Thus, if predictive global change models are to be developed it is essential that we build a clearer picture of how [CO<sub>2</sub>]-fertilisation, mineral nutrition and sink-controls on biomass production interact. To achieve this, we need a far better understanding of the molecular and genetic mechanisms that underpin the changes in biomass production and carbon allocation to rapid and slow turnover pools that occur in response to changing environmental factors.

In the experiments we present in this report, we have used the long-term cottonwood study carried out in the Intensive Forestry Mesocosm (IFM) of Biosphere 2 coupled to transcriptomics to analyse the changes in global gene expression induced by elevated [CO<sub>2</sub>] with the aim of identifying the genetic responses of both source leaves and stem sinks of poplar trees grown under a wide range of [CO<sub>2</sub>]. One of the primary advantages of the IFM experimental design was the ability to expose established, growing trees to three different, and constant, [CO<sub>2</sub>] making it possible to resolve changes in the transcriptome that might otherwise

have been hidden by a simpler pair-wise experiment, and by experiments in which the trees were exposed to elevated [CO<sub>2</sub>] only during the daylight hours. We present the first comprehensive analysis of gene expression in response to elevated CO<sub>2</sub> and discuss the differential patterns of expression in leaves and stems in terms of a mechanistic explanation of tree growth and carbon allocation patterns during long-term growth in elevated [CO<sub>2</sub>].

## Materials and methods

### *Plant material*

The experiment was performed in the Intensive Forestry Mesocosm (IFM) of the Biosphere 2 Laboratory located at Oracle, Arizona, USA in November 2002. The IFM was divided into three bays that were maintained at 400 (east), 800 (centre) and 1200  $\mu\text{mol mol}^{-1}$  CO<sub>2</sub> (west). Details of the structure, layout and control of environmental parameters have been described previously (Lin *et al.* 1998; Zabel *et al.* 1999; Barron-Gafford *et al.* 2005; Murthy *et al.* 2005). Cottonwood trees [*Populus deltoides* (Bartr.), genotype S7c8] originating from east Texas were grown for three seasons under continuously elevated [CO<sub>2</sub>]. At the end of each of the growing season the trees were chilled to induce dormancy and coppiced to 30 cm above soil level. In each subsequent growing season the trees re-grew from the stump and were pruned to one leader per tree. The growth and carbon partitioning responses of this agriforest system have been described elsewhere (Barron-Gafford *et al.* 2005).

This experimental system yielded unique material that had been grown and acclimatised over three full growing seasons to three different CO<sub>2</sub> concentrations, providing material suitable for assessing the impacts of long-term, stable changes in [CO<sub>2</sub>] on tree growth. Leaves and stems were collected at midday from three trees in each bay during the third week of November 2002. Although late in the season, all trees were still showing active growth with no terminal buds set at the time material was collected. The selected trees were evenly distributed in the bays in order to avoid any location-bias in the experiment. Leaves were collected at ~1.60 m above ground from all around the tree in order to eliminate light- and position-effects. For stem analyses, 10 cm long pieces were cut 15 cm down from the top of each tree. Samples were immediately frozen in liquid nitrogen and stored at -80°C.

### *Wood density and fibre analysis*

Wood density was measured in stem sections ~3 cm long and was calculated as the ratio of the dry weight to volume ( $\text{g cm}^{-3}$ ) (Oliveras *et al.* 2003). Additional small wood pieces from the same stems were chipped to provide matchstick-sized pieces for fibre analysis. These pieces were macerated (Kilpeläinen *et al.* 2003) and the reaction was neutralised by adding NaHCO<sub>3</sub>. The length and width of at least 100 intact fibres for each of the three replicates of each growth condition were measured with an Axioplan 2 microscope (Zeiss, Munich, Germany) connected to an image analysing system (AxioVision 3.1, Munich, Germany).

### *Microarrays*

Total RNA was isolated according to Chang *et al.* (1993) with the following modifications: no spermidine was used in the extraction buffer and 2.67% (v/v)  $\beta$ -mercaptoethanol was used instead of 2%. One additional extraction step was performed after LiCl precipitation, with the RNeasy kit (Qiagen Inc., Valencia, CA). The cDNA synthesis, probe preparation, hybridisation conditions, data collection and data analysis was performed according to Smith *et al.* (2004). The cDNA was synthesised from 20  $\mu\text{g}$  of total RNA as template and labelled with different fluorescence aminoallyl-dyes (Cy3 and Cy5) for microarray

hybridisations (Amersham Biosciences, Little Chalfont, UK). The samples were heated to 95°C for 3 min, chilled on ice for 30 s and applied to the ASP (automated slide processor, Amersham Lucidea SlidePro; Amersham Biosciences) chambers containing the prehybridised slides. The POP1 poplar microarray slides consisted of 13 490 EST cDNAs, spotted in duplicate, representing ~10 000 unique genes (Andersson *et al.* 2004). Each biological sample was hybridised three times with a dye-swap as a technical replicate. Arrays were scanned with a ScanArray 4000 (PerkinElmer Sverige AB, Sweden) at 543 nm and 633 nm wavelengths for the two fluorescence dyes Cy3 and Cy5 respectively at high resolution (5 µm). Image analysis was performed with GenePix Pro4.1 software (Axon Instruments, Sunnyvale, CA). Variation in gene expression was assessed with a Limma (Linear Models for Microarray data) package (Smyth 2004) in Bioconductor R 1.9.0 (<http://www.bioconductor.org>; verified 30 September 2005). Data were treated by print-tip loess normalisation after background subtraction (Edwards 2003) in Bioconductor (<http://www.bioconductor.org>). The changes in transcript abundance between plants grown in 400 µmol mol<sup>-1</sup> [CO<sub>2</sub>] and in elevated [CO<sub>2</sub>] (800 or 1200 µmol mol<sup>-1</sup>) were analysed by pairwise comparison. The statistical significance of the differences in expression was assessed using the B-statistic (Smyth 2004). In all analyses the differences in gene expression were considered significant only when they passed a statistically stringent filter of  $B \geq 10$ , and for those genes that passed the B-statistic filter, we then applied an additional cut-off of a change of more than 2-fold or less than -2-fold. For a better visualisation in GeneSpring 6.1 (SiliconGenetics, Palo Alto, CA) the data were analysed using the *in silico* values of the average transcript level from the six values for each clone (Diaz *et al.* 2003).

Supplementary material presented on the website of *Functional Plant Biology*

#### Appendix S1

Validation of reproducibility of the hybridisations by comparing the hybridisation results of three genes for which two independent cDNA clones had been spotted on the array. (A, C, E leaves; B, D, F stems). (A, B) Protein phosphatase 2A, 65 kDa subunit. (C, D) Elicitor-inducible cytochrome P450. (E, F) Major storage protein.

#### Appendix S2

[CO<sub>2</sub>]-responsive genes in leaves sorted according to functional categories, selected up-regulated clones under 800 or 1200 µmol mol<sup>-1</sup> [CO<sub>2</sub>]. Relative expression refers to fold changes between the two samples. Values printed in bold are statistically significant with B-statistic higher  $\geq 10$ .

#### Appendix S3

[CO<sub>2</sub>]-responsive genes in leaves sorted according to functional categories, selected down-regulated clones under 800 or 1200 µmol mol<sup>-1</sup> [CO<sub>2</sub>]. Relative expression refers to fold changes between the two samples. Values printed in bold are statistically significant with B-statistic higher  $\geq 10$ .

#### Appendix S4

[CO<sub>2</sub>]-responsive genes in stems sorted according to functional categories, selected up-regulated clones under 800 or 1200 µmol mol<sup>-1</sup> [CO<sub>2</sub>]. Relative expression refers to fold changes between the two samples. Values printed in bold are statistically significant with B-statistic higher  $\geq 10$ .

#### Appendix S5

[CO<sub>2</sub>]-responsive genes in stems sorted according to functional categories, selected down-regulated clones under 800 or 1200 µmol mol<sup>-1</sup> [CO<sub>2</sub>]. Relative expression refers to fold changes between the two samples. Values printed in bold are statistically significant with B-statistic higher  $\geq 10$ .

#### Appendix S6

Effect of long-term stable increases in [CO<sub>2</sub>] on the expression profile of energy-related genes in leaf and stem tissue. Relative expression refers to fold changes between the two samples. Values printed in bold are statistically significant with B-statistic higher  $\geq 10$ .

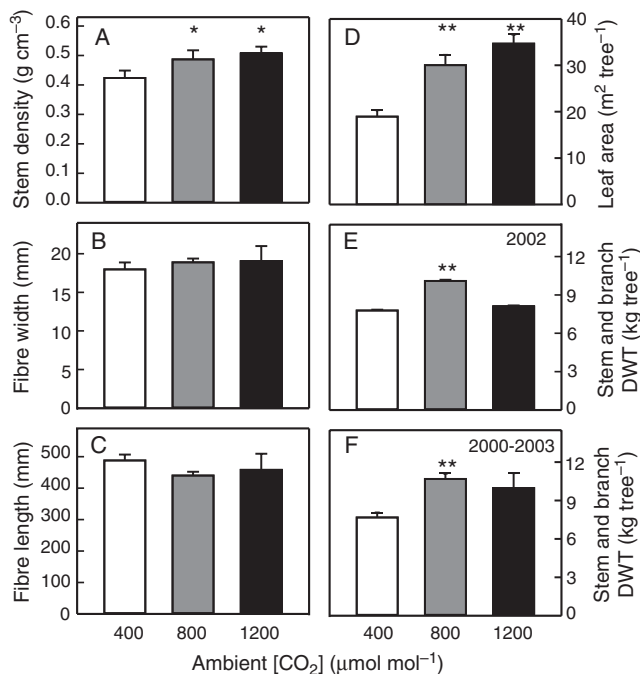
#### Appendix S7

Model of the effects of elevated [CO<sub>2</sub>] on the expression of genes of the flavonol biosynthesis pathway in leaves and stems of poplar. The coloured squares represent, from left to right, changes in gene expression at 400, 800 and 1200 µmol mol<sup>-1</sup> CO<sub>2</sub> respectively, relative to the *in silico* average of all samples from all treatments. 4CL, 4-coumarate-CoA ligase; C4H, trans-cinnamate 4-monooxygenase; CHI, chalcone isomerase; CHS, naringenin-chalcone synthase; DFR, dihydrokaempferol 4-reductase; F3H, naringenin 3-dioxygenase; F3'H, flavonoid 3'-monooxygenase; F3'5'H, flavonoid 3',5' hydroxylase; PAL, phenylalanine ammonia-lyase. The colour scale on the left depicts expression ratios in fold change.

## Results and discussion

### Stem biomass, density and fibre analysis

To characterise the effect of elevated [CO<sub>2</sub>] on the stem growth, we measured stem biomass accumulation for the 2002 growth season together with stem density, fibre length and fibre width from the samples harvested from the 400, 800, and 1200 µmol mol<sup>-1</sup> CO<sub>2</sub> IFM bays in November 2002. At increasing [CO<sub>2</sub>] the IFM cottonwood showed small but significant ( $P < 0.01$ ) increases in stem wood density (Fig. 1A), similar to that shown for both *Pinus radiata* (Atwell *et al.* 2003) and *Pinus sylvestris* (Kilpeläinen *et al.* 2003). We found no significant difference in fibre lengths or widths from stem tissues grown under elevated [CO<sub>2</sub>] but we did observe a trend to decreased fibre length (Fig. 1C) and increased fibre diameter (Fig. 1B), similar to that reported for several temperate broad-leaved species (Kaakinen *et al.* 2004). In the 2002 growing season, growth at 800 v. 400 µmol mol<sup>-1</sup> CO<sub>2</sub> also resulted in a significant ( $P < 0.01$ ) increase in stem and branch biomass (Fig. 1E), similar to that reported for *Pinus taeda* (DeLucia *et al.* 1999; Oren *et al.* 2001). However, this stimulation of biomass accumulation in stem and branch tissues (i.e. biomass that results from secondary stem growth) was not carried over in 2002 in the 1200 µmol mol<sup>-1</sup> CO<sub>2</sub> treatment (Fig. 1E). Even when we compare average stem and branch biomass accumulation for the years 2000–2003 rather than just 2002 (Fig. 1F), only the 800 µmol mol<sup>-1</sup> treatment yielded a significant ( $P < 0.01$ ) increase in biomass relative to the 400 µmol mol<sup>-1</sup> treatment [see Barron-Gafford *et al.* (2005) for a comprehensive analysis of the different growing seasons]. This loss in response to CO<sub>2</sub>



**Fig. 1.** Effect of elevated  $[\text{CO}_2]$  on stem wood density (A), fibre properties (B, C) average leaf area per tree calculated on 12 October 2002 (D) and seasonal stem and branch biomass accumulation for 2002 (E) and the average for 2000–2003 (F). Data shown in (D–F) recalculated from Barron-Gafford *et al.* (2005) and Murthy *et al.* (2005). Statistically significant differences were assessed by one-way ANOVA and differences from control ( $400 \mu\text{mol mol}^{-1} \text{CO}_2$  treatment) were assessed by Dunnett Multiple Comparison Post-Test: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

enrichment at progressively higher  $[\text{CO}_2]$  is similar to that previously reported for *Salix* (Julkunen-Tiitto *et al.* 1993), and it may be related to the loss of wood increment enhancement by elevated  $[\text{CO}_2]$  resulting from altered allocation patterns in favour of leaf area growth [Fig. 1D, see also Murthy *et al.* (2005)] and fine root production (Norby *et al.* 2002, 2004; Barron-Gafford *et al.* 2005; Trueman and Gonzalez-Meler 2005).

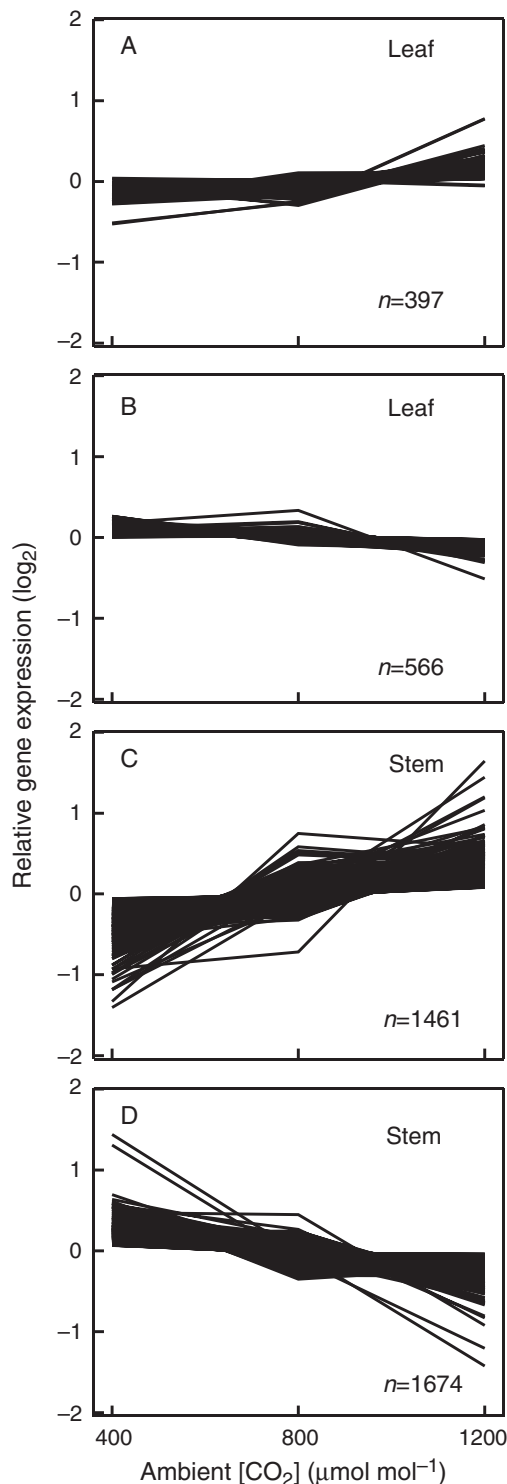
#### Microarray data

To assess the impact of long-term exposure to elevated  $[\text{CO}_2]$  on the transcriptomes of poplar leaves and stems, RNA was extracted from leaf and stem samples collected in November 2002, at a time when the trees were still exhibiting vigorous growth and before bud set and the onset of dormancy. Experimental replication included RNA extraction from three individual trees per treatment and three technical replicates per comparison. The expression profiles of three randomly selected genes for which independent ESTs had been spotted on the array confirmed the reproducibility of the hybridisations (Supplementary Appendix S1). When we analysed the expression data for the 13 490 clones present

on the POP1 arrays at all three experimental points and two different tissues, 95 and 277  $[\text{CO}_2]$ -responsive clones were found in leaves (Supplementary Appendices S2, S3) and stems (Supplementary Appendices S4, S5) respectively. Among them, 82 and 192 clones were up-regulated, 13 and 85 were down-regulated (in leaves and stems respectively). Changes in the numbers of  $[\text{CO}_2]$ -responsive genes with increasing  $[\text{CO}_2]$  indicate a stronger response at  $1200 \mu\text{mol mol}^{-1}$  in both leaves and stems and that the transcriptome of the stem was far more responsive to changing  $[\text{CO}_2]$  than that of the leaves (Fig. 2). The greater response of the stem and  $1200 \mu\text{mol mol}^{-1}$  transcriptomes is reflected by the increase in the number of genes related to transcription induced under these growth conditions (Tables 1, 2).

#### Functional categorisation of the $[\text{CO}_2]$ -responsive genes

Individual  $[\text{CO}_2]$ -responsive genes were assigned to 11 different functional categories with the MIPS database (MATDB; <http://mips.gsf.de/proj/thal/db>; verified 30 September 2005) with a *Populus*-modified version of EASE (Hosack *et al.* 2003). As might have been expected from this type of experiment, metabolism-related genes (Table 1) were the most responsive to elevated  $[\text{CO}_2]$  but interestingly, while both leaves and stems showed an enhancement of metabolism, only in the stems was there any evidence of strong down-regulation of expression of genes related to metabolism. Similarly, it is notable that no genes relating to energy processes showed any significant change in expression in leaves (Supplementary Appendix S6), indicating that in these experiments there was no response of the nuclear-encoded photosynthetic or respiratory transcriptomes to elevated  $[\text{CO}_2]$ . This is not surprising, because the enhancement of photosynthetic activity in *Populus deltoides* at the leaf level by elevated  $[\text{CO}_2]$  (Murthy *et al.* 2005) reflects the kinetics of the primary  $\text{CO}_2$  assimilation enzyme, Rubisco, often in spite of a reduction in the amount of enzyme protein following growth at elevated  $[\text{CO}_2]$  or following anti-sense interventions (Makino *et al.* 2000; Masle *et al.* 1993; Eichelmann *et al.* 2004). Furthermore, the acceleration of biomass production by elevated  $[\text{CO}_2]$  at the stand level in our experiments was largely attributable to increased foliar biomass and leaf area [Fig. 1D, see also Murthy *et al.* (2005)]. The trees sampled in the present experiment had almost twice the leaf area at elevated  $[\text{CO}_2]$  compared with  $400 \mu\text{mol mol}^{-1}$  (Fig. 1D). Acceleration of stand respiration by elevated  $[\text{CO}_2]$  was attributable to increased above-ground biomass, to rapid production of fine roots and their turnover at the end of the growing season, and the acceleration of soil microbial respiration (Barron-Gafford *et al.* 2005). It is now well established that elevated  $[\text{CO}_2]$  has no direct effect on respiratory processes, beyond those associated with increased substrate supply (Davey *et al.* 2004), and



**Fig. 2.** Gene tree clustering showing changes in gene expression with increasing [CO<sub>2</sub>] relative to the *in silico* average of all samples from all treatments. The four major clusters obtained by Self-Organising-Map in GeneSpring 6.1 (Silicon Genetics), representing up-regulated (A–C), down-regulated (B–D) clusters in leaves (A–B) and stems (C–D), expression ratios in log<sub>2</sub> scale. [CO<sub>2</sub>] is indicated at the bottom of the figures.

the lack of any noticeable effect of elevated [CO<sub>2</sub>] on energy metabolism genes in this experiment (Supplementary Appendix S6) supports this conclusion.

Looking at these broad changes more closely, increasing [CO<sub>2</sub>] to 800 μmol mol<sup>-1</sup> did not result in any major difference in the transcript profiles in leaves (Fig. 2). However, at 1200 μmol mol<sup>-1</sup> CO<sub>2</sub>, several genes related to metabolism and cell growth were up-regulated (Table 3). These changes predominantly represented an increase in expression of storage proteins but there was also an up-regulation of endo-xyloglucan transferase and xyloglucan endotransglycosylases (XET), both of which are involved in incorporating newly secreted xyloglucans into cell walls (Nishitani and Tominaga 1992; Wu and Cosgrove 2000). XET also has the ability to cleave xyloglucan chains and join the cut ends to new xyloglucan oligosaccharides, which may be important for cutting and rejoining the xyloglucan tethers between cellulose microfibrils, allowing for controlled wall expansion (Cosgrove 1997b; Wu and Cosgrove 2000). These data are consistent with earlier reports that elevated [CO<sub>2</sub>] may stimulate or prolong expansion in *Populus* leaves (Ceulemans *et al.* 1995; Gardner *et al.* 1995; Ferris *et al.* 2001; Taylor *et al.* 2003) and support the findings of enhanced growth activity in the early stages of leaf development, leading to an increase in final leaf size (Walter *et al.* 2005), increased leaf area [Fig. 1D, see also Murthy *et al.* (2005)] and increased leaf (but not stem) biomass (Barron-Gafford *et al.* 2005) in the IFM cottonwood growing at 1200 μmol mol<sup>-1</sup> CO<sub>2</sub>.

Interestingly, 3-deoxy-7-phosphoheptulonate synthase, which catalyses the condensation of phosphoenolpyruvate (PEP) with erythrose 4-phosphate (E4P), naringenin-chalcone synthase, chalcone isomerase and dihydrokaempferol 4-reductase are up-regulated in leaves at 1200 μmol mol<sup>-1</sup>, showing that the shikimic acid pathway and flavonol metabolism is enhanced in *Populus* leaves exposed to high [CO<sub>2</sub>] (Table 3; Supplementary Appendix S7), as previously shown for *Salix* (Julkunen-Tiitto *et al.* 1993) and birch (Lavola and Julkunen-Tiitto 1994) grown under elevated [CO<sub>2</sub>]. These data indicate that under high [CO<sub>2</sub>] the source/sink balance may be disturbed in fast-growing tree species, leading to alterations of carbon flow into flavonol metabolism. This alteration in carbon flow in source leaves could have important implications for future plant–herbivore interactions (Bezemer and Jones 1998; Jones *et al.* 1998; Bezemer *et al.* 2000), especially in light of recent findings that insect herbivory may be reduced at elevated [CO<sub>2</sub>] in intact temperate forest understoreys (Hamilton *et al.* 2004).

In contrast to the results from leaves, the genes up-regulated in the stems primarily represented enzymes directly related to lignin formation, such as CCoA-3H, COMT and CAD (Table 4; Fig. 3), and lignin polymerisation such as laccase (O'Malley *et al.* 1993; Ranocha *et al.* 1999, 2002)

**Table 1. Distribution of [CO<sub>2</sub>]-responsive genes organised by main functional categories**

Category	Up-regulated		Down-regulated	
	Leaves	Stems	Leaves	Stems
Transcription	2	13	1	0
Protein synthesis	0	2	0	0
Protein destination	3	3	1	2
Metabolism	13	18	2	15
Energy	0	3	0	0
Cellular organisation	1	1	1	2
Cellular communication and signalling	0	4	1	0
Cell rescue, defence, cell death, and ageing	1	5	0	1
Cell growth, cell division and ageing	3	10	0	2
Cell fate	0	1	1	0
Unclassified / classification not yet clear	28	42	4	17

**Table 2. Selection of [CO<sub>2</sub>]-responsive genes under 800 or 1200 µmol mol<sup>-1</sup> [CO<sub>2</sub>] belonging to the functional classes ‘Cell growth, cell division, DNA synthesis’ and ‘Transcription’ from leaves and stems**

Relative expression refers to fold changes between the two samples. Values printed in bold are statistically significant with B-statistic higher  $\geq 10$

Function	Clone ID	Gene ID	Relative expression		
			800 / 400	1200 / 400	1200 / 800
<b>Leaf</b>					
CCAAT-binding factor B subunit	PU03099	At1 g72830	-1.09	<b>2.12</b>	<b>2.30</b>
Probable coproporphyrinogen oxidase	PU03696	At3 g20740	-1.09	<b>2.30</b>	<b>2.51</b>
RGA1, gibberellin response modulation protein	PU03640	At2 g01570	-1.11	<b>2.20</b>	<b>2.44</b>
RING zinc finger ankyrin protein-related	PU03201	At2 g28840	-1.12	<b>1.85</b>	<b>2.08</b>
Protein phosphatase 2A 65 kDa regulatory subunit	PU05097	At3 g25800	2.52	<b>2.66</b>	<b>2.23</b>
<b>Stem</b>					
CCCH-type zinc finger protein	PU10193	At2 g40140	<b>2.28</b>	<b>4.38</b>	<b>1.92</b>
GTP-binding protein	PU08983	At5 g55190	<b>2.00</b>	<b>2.75</b>	<b>1.37</b>
SOS2-like protein kinase PKS2	PU07078	At5 g58380	1.78	<b>3.10</b>	1.74
Transcription factor BHLH12	PU06496	At4 g00480	-1.93	1.28	<b>2.47</b>
bZip transcription factor (AtbZip60)	PU13464	At1 g42990	1.40	<b>2.46</b>	<b>1.76</b>
CONSTANS B-box zinc finger family protein	PU08816	At3 g21150	1.72	<b>2.70</b>	1.57
DNA-binding protein, putative	PU13388	At1 g01060	<b>2.57</b>	<b>2.35</b>	-1.09
Ethylene responsive element binding factor 4	PU07741	At3 g15210	<b>1.93</b>	<b>3.39</b>	<b>1.75</b>
Ethylene-responsive transcriptional coactivator-like protein	PU03794	At3 g24500	<b>4.67</b>	<b>7.39</b>	1.58
Homeobox-leucine zipper protein ATHB-12	PU09347	At3 g61890	1.30	<b>4.15</b>	<b>3.19</b>
SCARECROW gene regulator-like	PU07055	At5 g48150	1.05	<b>2.14</b>	<b>2.03</b>
Transcription factor WRKY4	PU09504	At1 g80840	1.22	<b>2.05</b>	<b>1.68</b>
WRKY-type DNA binding protein	PU09430	At2 g46400	3.81	<b>9.23</b>	2.42

**Table 3. Selection of [CO<sub>2</sub>]-responsive genes under 800 or 1200 µmol mol<sup>-1</sup> [CO<sub>2</sub>] from leaves**

Analyses were done by pair wise comparison between [CO<sub>2</sub>]. Relative expression refers to fold changes between the two samples. Values printed in bold are statistically significant with B-statistic higher  $\geq 10$

Function	Clone ID	Gene ID	Relative expression		
			800 / 400	1200 / 400	1200 / 800
Major storage protein	PU03513	At4 g24340	-0.80	<b>2.42</b>	<b>2.73</b>
Major storage protein	PU03509	At3 g17650	-1.09	<b>2.34</b>	<b>2.57</b>
Xyloglucan endotransglycosylase	PU02379	At4 g03210	-0.90	<b>2.09</b>	<b>2.32</b>
Endo-xyloglucan transferase	PU11514	At5 g65730	1.17	<b>2.00</b>	<b>1.70</b>
Cellulase	PU03243	At3 g26130	-1.02	<b>2.42</b>	<b>2.47</b>
Dihydrokaempferol 4-reductase	PU12089	At1 g61720	1.03	<b>2.22</b>	<b>2.15</b>
Naringenin 3-dioxygenase	PU01480	At3 g51240	-1.01	<b>1.70</b>	1.71
Naringenin-chalcone synthase	PU10149	At5 g13930	1.34	<b>1.67</b>	1.23
Chalcone isomerase	PU04986	At3 g55120	1.23	<b>1.45</b>	1.28
3-Deoxy-7-phosphoheptulonate synthase	PU02218	At1 g22410	1.06	<b>2.28</b>	<b>2.21</b>

**Table 4. Selection of [CO<sub>2</sub>]-responsive genes under 800 or 1200 μmol mol<sup>-1</sup> [CO<sub>2</sub>] from stems related to lignin and flavonol biosynthesis**  
Analyses were done by pair wise comparison between [CO<sub>2</sub>]. Relative expression refers to fold changes between the two samples. Values printed in bold are statistically significant with B-statistic higher ≥ 10

Function	Clone ID	Gene ID	Relative expression		
			800 / 400	1200 / 400	1200 / 800
<b>Lignin biosynthesis</b>					
Caffeate <i>O</i> -methyltransferase	PU07978	At5 g54160	1.45	<b>2.41</b>	1.63
Cinnamyl-alcohol dehydrogenase	PU00508	At3 g19450	1.49	<b>2.38</b>	<b>1.59</b>
Caffeoyl-CoA <i>O</i> -methyltransferase	PU02461	At4 g34050	1.85	<b>2.37</b>	1.29
Laccase	PU02804	At2 g38080	-1.17	<b>2.10</b>	<b>2.46</b>
Putative basic chitinase	PU06113	At3 g16920	1.28	<b>3.37</b>	<b>2.63</b>
Ethylene responsive element binding factor 4	PU07741	At3 g15210	<b>1.93</b>	<b>3.39</b>	<b>1.75</b>
Ethylene responsive element binding factor	PU13199	At4 g17490	<b>3.50</b>	<b>5.33</b>	1.52
Ethylene-responsive transcriptional co-activator	PU03794	At3 g24500	<b>4.67</b>	<b>7.39</b>	1.58
<b>Flavonol biosynthesis</b>					
Naringenin-chalcone synthase	PU12625	At5 g13930	-1.35	<b>-4.03</b>	<b>-2.99</b>
Chalcone isomerase	PU04986	At3 g55120	-1.52	<b>-2.07</b>	-1.36
Naringenin 3-dioxygenase	PU01480	At3 g51240	-1.48	<b>-3.16</b>	<b>-2.14</b>
Flavonoid 3'-monooxygenase	PU12061	At5 g07990	-1.53	<b>-2.75</b>	<b>-1.8</b>
Dihydrokaempferol 4-reductase	PU12089	At1 g61720	-1.59	<b>-4.23</b>	<b>-2.66</b>
Leucoanthocyanidin reductase	PU12098	At4 g22880	-1.66	<b>-3.51</b>	<b>-2.12</b>

and other lignin-related genes such as a putative basic chitinase (Table 4) (Zhong *et al.* 2002). We also show an increase in the abundance of ethylene response factors under elevated [CO<sub>2</sub>] (Table 4), which are known to induce lignin biosynthesis (Sitbon *et al.* 1999). These changes in transcript abundance for genes involved in lignification became more pronounced with increasing [CO<sub>2</sub>] (Fig. 3). Concomitant with this enhancement of pathways for lignin biosynthesis, there was also a pronounced repression of genes related to cell wall formation and cell growth (Table 5) and for flavonol biosynthesis (Fig. 3). The repression of genes such as XET and expansin (Cosgrove 1997a; Hutchison *et al.* 1999) in stems under high [CO<sub>2</sub>] is the opposite of what was observed in leaves, indicating very different responses of cell growth to [CO<sub>2</sub>] in source and sink tissues (Table 2). The microarray data set also contained a large number of unknown genes, and functional analysis of these genes will provide deeper insight into the regulation and processes involved in woody plant responses to CO<sub>2</sub> enrichment.

When the above data are considered together with the reduced expression of genes responsible for the production of precursors for the cell wall (GDP-mannose pyrophosphorylase) (Keller *et al.* 1999; Lukowitz *et al.* 2001), of structural proteins (proline-rich protein) (Bernhardt and Tierney 2000; Fowler *et al.* 1999) and the decrease in the activity of pectin esterase, which leads to a reduction in the acidic pectin and then to a decrease in the stimulation of the enzymes involved in expansion of the cell wall (Darley *et al.* 2001; Hasunuma *et al.* 2004), it provides strong evidence that the production and expansion of

cell walls is compromised in poplar stems growing at high [CO<sub>2</sub>]. This conclusion is supported by the structural data showing a trend toward decreasing fibre length with increasing [CO<sub>2</sub>] (Fig. 1). At both 800 and 1200 μmol mol<sup>-1</sup> CO<sub>2</sub> there was also a general decrease in expression of several genes encoding enzymes in flavonol synthesis (Table 4), again in contrast to what was seen in leaves. The pathways of flavonol biosynthesis may be down-regulated at high [CO<sub>2</sub>] to compensate for the strong induction of lignin biosynthesis, which represents one of the most carbon skeleton-demanding biosynthetic pathways in tree stems (Rogers and Campbell 2004).

These effects on flavonol and lignin biosynthetic pathways by elevated [CO<sub>2</sub>] noted above are different from the recent study (Kaakinen *et al.* 2004) showing significant increases in lignin content in aspen growing in the long-term Aspen-FACE experiment in response to ozone fumigation but not to elevated [CO<sub>2</sub>]. However, the Aspen-FACE experiment only exposed the trees to a 200 μmol mol<sup>-1</sup> increase in [CO<sub>2</sub>] during the daylight hours, and it is probable we would not have detected significant changes in transcript abundance in our experiment under such a scenario. These changes in lignin biosynthetic enzymes in the IFM experiment correlate with the higher wood density of trees growing at both 800 and 1200 μmol mol<sup>-1</sup> CO<sub>2</sub>, and the negative correlation with growth rates in the IFM experiment is consistent with earlier reports of antisense reduction of 4CL (see Fig. 3) activity reducing lignin content and enhancing growth in transgenic *Populus tremuloides* (Hu *et al.* 1999). Furthermore, up-regulation of lignin biosynthetic genes has been shown in a recent transcriptome



**Table 5. Selection of [CO<sub>2</sub>]-responsive genes under 800 or 1200 μmol mol<sup>-1</sup> [CO<sub>2</sub>] from stems related to cell wall formation**

Analyses were done by pair wise comparison between [CO<sub>2</sub>]. Relative expression refers to fold changes between the two samples. Values printed in bold are statistically significant with B-statistic higher ≥ 10

Function	Clone ID	Gene ID	Relative expression		
			800/400	1200/400	1200/800
Xyloglucan endotransglycosylase	PU05203	At4 g03210	-2.16	<b>-2.40</b>	1.15
Pollen allergen	PU01133	At4 g38400	-2.09	<b>-2.42</b>	1.31
GDP-mannose pyrophosphorylase	PU03161	At2 g39770	-1.36	<b>-2.25</b>	-1.66
Proline-rich cell wall protein	PU08800	At2 g14890	-1.35	<b>-2.17</b>	<b>-1.6</b>
Pectin esterase	PU01896	At3 g49220	-1.17	<b>-2.40</b>	<b>-2.05</b>
Expansin-like protein	PU05915	At1 g69530	1.97	-1.14	<b>-2.24</b>

study to correlate with the reduced stem biomass production by various *Eucalyptus* hybrids grown under ambient [CO<sub>2</sub>] (Kirst *et al.* 2004). Our results suggest that high [CO<sub>2</sub>]-induced lignin biosynthesis may be part of the explanation for the loss of CO<sub>2</sub>-enrichment response in stem and branch biomass production in the IFM experiment [Fig. 1A; Barron-Gafford *et al.* (2005)]. However, this may also have been augmented by changing biomass allocation patterns favouring fine root production (Norby *et al.* 2004; Barron-Gafford *et al.* 2005). Whether this change in allocation was driven by increased root sink strength or increased carbon supply to the roots because of restricted stem growth owing to increased lignification is unclear and warrants further investigation.

### Conclusions and perspectives

It is clear from the *Populus deltoides* experiments examined here that elevated [CO<sub>2</sub>] did not promote sequestration of carbon, even on the rich soils supporting these experimental agriforests, but instead promoted more rapid soil respiration and actually stimulated the respiration of 'old carbon' reserves in the soil (Barron-Gafford *et al.* 2005; Trueman and Gonzalez-Meler 2005). Furthermore, the changes in stem wood density in response to elevated [CO<sub>2</sub>] observed for *Populus deltoides* in this experiment, which are similar to those reported for other broad-leaved species (Kaakinen *et al.* 2004) and for *Pinus spp.* (Atwell *et al.* 2003; Conroy *et al.* 1990a), when combined with the differential regulation of biochemical pathways and genes involved in cell wall formation and expansion, have clear consequences not only for carbon sequestration but also for wood quality for both timber and paper production. Characterisation of such differentially expressed genes in source leaves and stem sinks is an initial step toward understanding acclimation to elevated [CO<sub>2</sub>] and can provide the insights needed for a more targeted approach in the future.

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