Transcriptomic comparison in the leaves of two aspen genotypes having similar carbon assimilation rates but different partitioning patterns under elevated [CO_2]

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Summary

- This study compared the leaf transcription profiles, physiological characteristics and primary metabolites of two *Populus tremuloides* genotypes (clones 216 and 271) known to differ in their responses to long-term elevated [CO_2] (e[CO_2]) at the Aspen free-air CO_2 enrichment site near Rhinelander, WI, USA.
- The physiological responses of these clones were similar in terms of photosynthesis, stomatal conductance and leaf area index under e[CO_2], yet very different in terms of growth enhancement (0–10% in clone 216; 40–50% in clone 271). Although few genes responded to long-term exposure to e[CO_2], the transcriptional activity of leaf e[CO_2]-responsive genes was distinctly different between the clones, differentially impacting multiple pathways during both early and late growing seasons.
- An analysis of transcript abundance and carbon/nitrogen biochemistry suggested that the CO_2-responsive clone (271) partitions carbon into pathways associated with active defense/response to stress, carbohydrate/starch biosynthesis and subsequent growth. The CO_2-unresponsive clone (216) partitions carbon into pathways associated with passive defense (e.g. lignin, phenylpropanoid) and cell wall thickening.
- This study indicates that there is significant variation in expression patterns between different tree genotypes in response to long-term exposure to e[CO_2]. Consequently, future efforts to improve productivity or other advantageous traits for carbon sequestration should include an examination of genetic variability in CO_2 responsiveness.

Introduction

Atmospheric CO_2 concentration ([CO_2]) has risen by ~30% in the last 250 yr, and data from monitoring stations, together with historical records extracted from ice cores, show that atmospheric [CO_2] is now at a level higher than at any time in the last 650 000 yr (Meehl *et al.*, 2007). Driven by the addition of 6–8 Pg carbon yr^{-1} from anthropogenic sources, atmospheric [CO_2] is predicted to continue to rise by an additional 50% by 2050 (Meehl *et al.*, 2007). The Intergovernmental Panel on Climate Change (IPCC) estimates that between 46 and 56% of terrestrial carbon stocks are found in forest biomes, and that actions to preserve and enhance this carbon sink would probably increase the global terrestrial carbon stock by 60–87 Pg carbon by 2050, thereby offsetting *c.* 15% of the anthropogenic emissions predicted for the same period (Prentice, 2001; Brown, 2002).

Aggressive afforestation is part of the action required to meet this potential for increasing the terrestrial carbon stock, and managed plantations of highly productive tree species, such as members of the genus *Populus*, are an attractive system to help achieve this goal (Deckmyn *et al.*, 2004; Perlack *et al.*,

Dedication: This paper is published in memory of Dr David F. Karnosky, Professor, School of Forest Resources and Environmental Science, Michigan Technological University and director of the Aspen FACE site.

2005). Recent advances in poplar genomic resources include the collections of over 410 000 expressed sequence tags (ESTs) in GenBank (http://www.ncbi.nlm.nih.gov/dbEST/), the development of cDNA and whole-genome microarrays (Déjardin *et al.*, 2004; Brosché *et al.*, 2005; Rinaldi *et al.*, 2007) and the full genome sequence available from *P. trichocarpa* (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home; Tuskan *et al.*, 2006). These advances have promoted the idea that poplar species hold great potential to be bred or engineered for an increased suitability for carbon sequestration (Sims *et al.*, 2006).

Plants, including trees, sense and respond to increasing [CO_2] through increased photosynthesis and reduced stomatal conductance (Ainsworth & Rogers, 2007; Hyvönen *et al.*, 2007; Liberloo *et al.*, 2007; Riikonen *et al.*, 2008; Taylor *et al.*, 2008). All other effects of elevated [CO_2] (e[CO_2]) on plants and ecosystems are derived from these two fundamental responses. However, genetic and environmental bottlenecks can determine both the magnitude of these primary responses to e[CO_2] and the capacity to assimilate carbon into increased above-ground biomass (Karnosky *et al.*, 2005; Liberloo *et al.*, 2006; Luo *et al.*, 2006, 2008; Ainsworth & Rogers, 2007). In trees, growth is dependent on the internal balance between carbon and nitrogen, and this balance depends not only on nitrogen resources directly available from soil and internal sources, but also on nitrogen resources from seasonal storage pools in bark, wood and roots (Luo *et al.*, 2008). A few studies have explored the transcriptomics of individual tree species as they respond to e[CO_2] (Gupta *et al.*, 2005; Taylor *et al.*, 2005; Druart *et al.*, 2006). Each study identified relatively few transcriptional changes in response to e[CO_2], and the results between species were quite variable, making it difficult to draw solid conclusions. Although the responses of photosynthesis, growth and biomass accumulation in trees grown at e[CO_2] are well documented (Nowak *et al.*, 2004; Ainsworth & Long, 2005; Norby *et al.*, 2005; Ainsworth & Rogers, 2007), the molecular mechanisms that determine how different tree species achieve a balance between carbon and nitrogen assimilation, storage and eventual growth remain largely unknown. Consequently, there is a need to increase our understanding of metabolic/physiological processes that may limit the response of trees to increasing [CO_2].

Free-air CO_2 enrichment (FACE) provides a realistic platform on which to investigate the response of trees to e[CO_2] (Long *et al.*, 2004) and, with particular reference to this study, avoids artificial restriction of sink capacity, canopy development and nutrient supply. In this study, we have taken advantage of an ongoing FACE experiment located near Rhinelander, WI, USA (Karnosky & Pregitzer, 2006), referred to as the Aspen FACE site (http://aspenface.mtu.edu/). Here, five distinct genotypes of quaking aspen (*Populus tremuloides*) have been grown at e[CO_2] (ambient plus 200 ppb) since 1997.

Poplar trees growing under e[CO_2] generally have a larger leaf size (Oksanen *et al.*, 2001; Riikonen *et al.*, 2008), increased stem and branch growth (Isebrands *et al.*, 2001; Karnosky *et al.*, 2005; King *et al.*, 2005; Kubiske *et al.*, 2007) and increased root biomass (King *et al.*, 2001; Lukac *et al.*, 2003). However, at Aspen FACE, CO_2 responsiveness was found to be genotype dependent. Clone 271 is highly responsive to e[CO_2], whereas the growth of clone 216 is not significantly stimulated by e[CO_2], despite the fact that both clones show similar increases in photosynthetic rates under e[CO_2] (Noormets *et al.*, 2001b; Riikonen *et al.*, 2008; Taylor *et al.*, 2008). Clone 271 has delayed senescence and develops ~50% more stem biomass than clone 216 in response to e[CO_2] (Table 1) (Karnosky *et al.*, 2005; Kubiske *et al.*, 2007). By focusing on the tissues that most directly sense and respond to e[CO_2], this paper provides an initial examination of the abundance of leaf gene transcripts and biochemical responses in growth-responsive (clone 271) and growth-unresponsive (clone 216) genotypes of *P. tremuloides* grown at ambient and e[CO_2]. It is hypothesized that such an analysis of clones with markedly different growth responses to e[CO_2] will allow us to identify some of the genetic trends associated with a sustained utilization of an increased carbon supply and a superior capacity for above-ground biomass production. To our knowledge, this is the first study to directly compare the leaf transcriptomes of tree genotypes of the same species having similar carbon uptake, but very different growth responses when grown at e[CO_2] in the field.

Materials and Methods

Experimental site and plant material

The study was performed on leaf tissues collected near Rhinelander, WI, USA (89.5°W, 45.7°N) at the Aspen FACE experimental site, which has been operating since 1997. The design and operation of this experimental site have been described in detail elsewhere (Dickson *et al.*, 2001; Karnosky *et al.*, 2005; Karnosky & Pregitzer, 2006). In short, 12, 30-m diameter, plots are contained within a 32-ha site in a full factorial design. The plots are exposed to ambient air (control) and air enriched with CO_2, ozone, or CO_2 and ozone in combination. In this study, only plots receiving ambient air ([CO_2] = 372 μl l^{-1}) and air enriched with CO_2 (target [CO_2] = 560 μl l^{-1}) were used. The site is divided into three blocks, each containing a CO_2 and a control plot. e[CO_2] plots were fumigated from sunrise to sunset for the length of the growing season (May–September), and 1-min averages of [CO_2] in the e[CO_2] plots were within 20% of the target concentration. The soil at the Aspen FACE site is a sandy loam and is characteristic of a moderate to poor soil for the region. Nitrogen is considered to be the principal limiting nutrient, and no supplemental fertilizer has been added (Ellsworth *et al.*, 2004; Karnosky & Pregitzer, 2006).

As a result of the significant differences in growth response to e[CO_2], this study utilized two trembling aspen (*Populus*

Table 1 Summary of physiological changes in response to long-term elevated [CO_2] (e[CO_2]) in aspen (*Populus tremuloides*) clones 216 and 271

	Clone 216 response to e[CO_2]	Clone 271 response to e[CO_2]	Reference(s)
Leaf criteria			
Photosynthesis	+++	+++	Noormets *et al*. (2001b); Riikonen *et al*. (2008); Taylor *et al*. (2008)
Chlorophyll content	——	——	Wustman *et al*. (2001)
Rubisco	—	ns	Wustman *et al*. (2001)
Stomatal conductance	—	—	Noormets *et al*. (2001a); Riikonen *et al*. (2008)
Stomatal frequency	ns	ns	Karnosky *et al*. (2003); Mankovska *et al*. (2005)
Leaf area index (LAI)	++	++	Karnosky *et al*. (2005) Riikonen *et al*. (2008); Taylor *et al*. (2008)
Leaf thickness	++	++	Oksanen *et al*. (2001, 2003)
Leaf cell wall thickness	ns	—	Oksanen *et al*. (2003)
Leaf surface waxes	ns	++	Karnosky *et al*. (2002); Mankovska *et al*. (2005)
Leaf isoprene emissions	ns	ns	Calfapietra *et al*. (2007)
Leaf cytoplasmic lipids	++	+	Oksanen *et al*. (2001)
Leaf starch content	++	++	Kopper & Lindroth (2003)
Leaf total nitrogen	—	—	Kopper & Lindroth (2003)
Rust occurrence	+	ns	Karnosky *et al*. (2002); Mankovska *et al*. (2005)
Tent caterpillar performance	ns	—	Kopper & Lindroth (2003)
Delayed autumn senescence	+	++	Riikonen *et al*. (2008); Taylor *et al*. (2008)
Stem/wood criteria			
Diameter growth	+	+++	Isebrands *et al*. (2001); Kubiske *et al*. (2007)
Volume growth	+	+++	Karnosky *et al*. (2005); Kubiske *et al*. (2007)
Height growth	ns	+++	Isebrands *et al*. (2001); Kubiske *et al*. (2007)
Cell wall percentage	+	ns	Kaakinen *et al*. (2004)
Wood starch content	ns	+	Kaakinen *et al*. (2004)
Wood total nitrogen	–	—	Kaakinen *et al*. (2004)

ns, no significant response; +, trend of increase (< 10%); –, trend of decrease (< 10%); ++, significant increase (10–30%); —, significant decrease (10–30%); +++, large and significant increase (> 30%); ——, large and significant decrease (> 30%).

tremuloides Michx.) genotypes: clone 216 and clone 271. To avoid variation derived from different leaf emergence dates on indeterminate shoots, leaves were sampled from short shoots. Short shoots have a determinate growth form and all leaves expand rapidly at the same time, at the start of the growing season (Cox, 2005). Likewise, to avoid variation resulting from leaf developmental stage, only mature, fully expanded leaves were used for analysis. As bud-break in this region occurred on 19 May and bud-set occurred in late August (for clone 216) and early September (for clone 271), harvest dates of 15 June and 17 August 2005 were chosen to ensure recently mature early-season leaf collections and mature, but not yet senescing, leaf collections in the late-season time point. Upper canopy sunlit leaves were harvested between 10:00 and 11:00 h on each date. Tissues were frozen in liquid nitrogen within 20 s of harvesting and stored at −80°C until analysis. To avoid potential variation derived from time-of-day effects, all pairs of control vs. e[CO_2] samples were collected within 10 min of one another.

Physiological data collection

Stem volume growth comparisons of aspen clones 216 and 271 in response to e[CO_2] were collected for 8 yr, as described previously (Karnosky *et al*., 2003; Kubiske *et al*., 2007). Maximum instantaneous photosynthesis (A_{max}) of aspen clones 216 and 271 was compared under ambient and e[CO_2] using an LI-6400 portable photosynthesis system (Li-Cor Inc., Lincoln, NE, USA), and the data were analyzed as described previously (Noormets *et al*., 2001a,b). Data were expressed as the mean of three leaves each from three trees measured during midday and replicated three times (6, 7 and 11 July 2006) from the full-sun canopy.

cDNA microarrays

The PICME (Platform for Integrated Clone Management; http://www.picme.at) *Populus* microarrays are composed of ~28 000 elements, including 6528 ESTs of *Populus interamericana* from INRA-Nancy (Rinaldi *et al*., 2007), 12 202 ESTs of *Populus alba* × *Populus tremula* from INRA-Orleans (Déjardin *et al*., 2004) and 8160 cDNA clones of *Populus euphratica* from the University of Helsinki (Brosché *et al*., 2005). This set of leaf, root and xylem cDNAs corresponds to ~10 000 different predicted gene models in the *P. trichocarpa* genome sequence (Tuskan *et al*., 2006; Rinaldi *et al*., 2007). The arrays are well documented for leaf transcript analysis (Rinaldi *et al*., 2007; Fluch *et al*., 2008) and are fully described

under platform GPL6036 stored in the Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/geo).

We followed the widely accepted Minimum Information About a Microarray Experiment (MIAME) guidelines for microarray analysis and verification (Zimmermann *et al.*, 2006). For each of the two-channel arrays, RNA collected from plants grown in the ambient (control) ring was compared directly with RNA derived from the same genotype grown under e[CO_2], following cDNA synthesis and labeling procedures in the Superscript™ Indirect cDNA Labeling System (Invitrogen, Carlsbad, CA, USA). Three independent biological replicates, derived from trees grown in three independent replicate FACE rings, were analyzed using equal amounts (40 pmol) of labeled samples. In addition, each clone and time point included dye swap reciprocal two-color experiments for each biological replicate (Churchill, 2002; Allison *et al.*, 2006). Thus, six data points per cDNA (three biological replicates with two technical replicates each) were used.

Slides were scanned using a VersArray ChipReader™ scanner (BioRad, Hercules, CA, USA) at 5-μm resolution. Cyanine-3 (Cy3) and cyanine-5 (Cy5) images were aligned and spots were flagged using VersArray Analyzer 5.0 (BioRad). After local background subtraction, the signal intensity was $\log_2$ transformed and normalized by the LOWESS algorithm with a smoothing parameter of 0.2, using GeneGazer software (Bio-Rad). Normalized intensity values were filtered by a coefficient of variance (CV) cut-off of 0.25, and spots having intensities below 100 in both channels were also excluded from further analysis. Filtered gene lists were subjected to *t*-tests with a false discovery rate at 0.05 ($P < 0.05$) (see Fig. S1, Supporting Information, for examples of data linearity). Principal component analysis (PCA) was conducted using standard correlation and hierarchical cluster analysis (HCA) was performed using the Euclidean distance metric and average cluster linkage. The complete expression dataset, sample details, RNA extraction, cDNA preparations, labeling of cDNAs and hybridization procedures are available with the series accession number GSE14881 in GEO at NCBI.

Quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

DNA-free total RNA was used for cDNA synthesis with oligo-dT primers, as described previously; however, it is important to note that independent RNA preparations were used for quantitative RT-PCR analyses. In this way, the quantitative RT-PCR samples represent additional biological replicates from the same field sampling. EST sequences for genes of interest from the microarray analysis were retrieved from GenBank and used to identify their cognate gene models from the Joint Genome Initiative (JGI) *Populus* genome portal. Because a large fraction of *Populus* genes are derived from genome-wide duplication (Tuskan *et al.*, 2006), closely related gene family members were aligned in order to evaluate the likelihood of cross-hybridization on the EST arrays. Depending on sequence homology, gene- or group-specific primers (Table S1, see Supporting Information) flanking 137–290-bp amplicons near the 3′-untranslated regions (3′-UTRs) were designed on the basis of JGI-predicted cDNA sequences and the corresponding GenBank *Populus* EST sequences.

Real-time PCRs were performed using the Mx3000P Real-Time PCR System (Stratagene, La Jolla, CA, USA) with the ABsolute QPCR SYBR Green Mixes (ABgene Inc., Rochester, NY, USA). The PCR parameters were as follows: 15 min at 95°C, followed by 40 cycles of 15 s at 95°C, 1 min at 58–66°C (Table S1) and 1 min at 72°C. Each reaction was performed in duplicate with three biological replicates (from replicate rings) and no-template controls, using cDNA synthesized from 2.5 ng of total RNA. Because the housekeeping genes were expressed relatively strongly in these tissues, PCR amplification was performed using diluted cDNA (200-fold for cyclophilin and ubiquitin-conjugating enzyme E2, and four-fold for actin) in order to improve relative transcript abundance estimates of weakly expressed genes. The specificity of the amplification was verified using dissociation curve analysis at the end of each run, employing MxPro software (Stratagene). The threshold level was manually adjusted and applied consistently across all amplifications to obtain the threshold cycle (C_T) values. Relative target transcript levels were normalized to the geometric mean of three housekeeping genes using the ΔC_T method (Tsai *et al.*, 2006).

Biochemical analysis

Frozen leaf tissues, derived from the same tissue collections as described above, were powdered in liquid nitrogen and transferred to microcentrifuge tubes; 375 μl of 80% (v/v) ethanol, 10 mM 2-(*N*-morpholino)ethanesulphonic acid (MES), pH 6.0 was added to equal quantities of tissue. The tubes were mixed thoroughly and incubated at 80°C for 30 min. Extracts were clarified by centrifugation (4500 ***g***, 10 min), and the supernatant was decanted into a 96-well, deep-well microplate, stored at 4°C; 225 μl of 80% (v/v) ethanol, 10 mM MES pH 6, and 375 μl of 50% (v/v) ethanol, 10 mM MES pH 6, were added in two additional, separate incubations to the pellet and supernatants pooled in the deep-well plate.

Glucose, fructose, sucrose and starch content were determined from the supernatant and pellet resulting from the ethanol extract using the continuous enzymatic substrate assay described by Rogers *et al.* (2006). The total amino acid content in the ethanol extract was determined using a fluorogenic-based microplate assay, as described previously (Rogers *et al.*, 2006). Protein content was determined using a commercial kit (Pierce, Rockford, IL, USA) as described previously (Ainsworth *et al.*, 2007). Nitrate was determined by conversion to nitrite by nitrate reductase and subsequent colorimetric determination

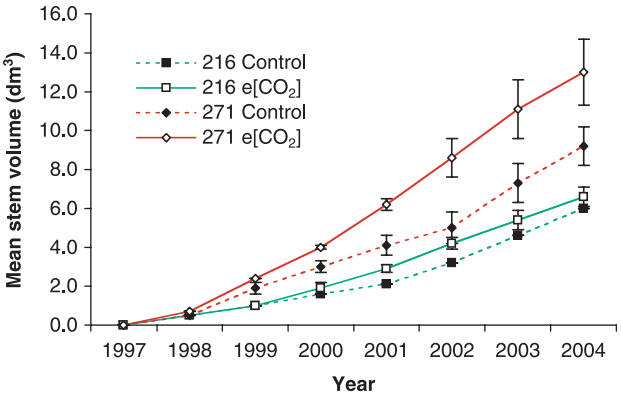

Fig. 1 Annual stem volume growth (mean ± SE) comparison of aspen (*Populus tremuloides*) clones 216 and 271 in response to long-term elevated [CO_2] (e[CO_2]).

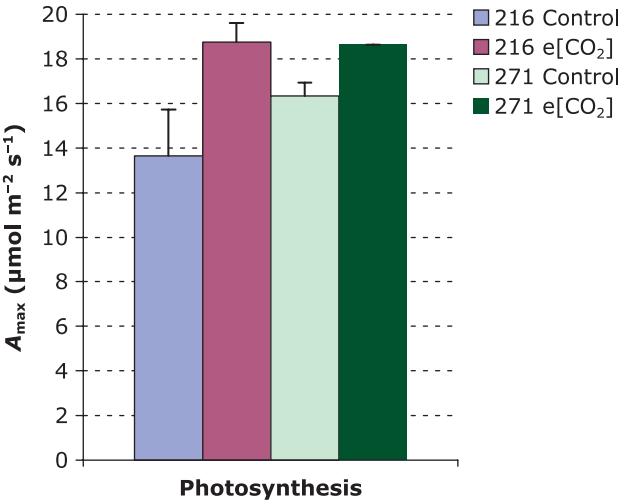

Fig. 2 Maximum light-saturated photosynthesis (A_{max}) of aspen (*Populus tremuloides*) clones 216 and 271 compared under ambient and long-term elevated [CO_2] (e[CO_2]). Data are expressed as the means ± SE of three leaves each from three trees.

by the Griess reaction (Cross *et al.*, 2006). Biochemical statistical analyses were based on the treatment plot as the experimental unit. Values for three leaves per clone per plot (subsamples) were averaged to provide the sample estimate for each treatment plot ($n = 3$ blocks). The experimental design was a split-split-plot with [CO_2] as the main plot factor. Clone was nested within [CO_2] as a split-plot factor and date was the split-split-plot factor. A mixed model ANOVA was used for statistical analysis (SYSTAT, SPSS Inc., Chicago, IL, USA, 2000).

Results and Discussion

Physiological measurements alone do not explain the superior ability of clone 271 to allocate carbon towards growth, whilst clone 216 remains largely unresponsive under e[CO_2] conditions

e[CO_2] has been shown to increase photosynthesis in plants, including trees (Ainsworth & Long, 2005; Hyvönen *et al.*, 2007), largely through increases in Rubisco-limited photosynthesis in leaf tissues, the tissues that most directly sense and respond to e[CO_2] (Wustman *et al.*, 2001; Long *et al.*, 2004; Ainsworth & Rogers, 2007). At the Aspen FACE site (http://aspenface.mtu.edu/), photosynthetic enhancement has ranged from 10 to 80% depending on the tree species and seasonal variation (Noormets *et al.*, 2001b; Riikonen *et al.*, 2008; Taylor *et al.*, 2008). This enhancement translated into an increase in leaf and stem carbohydrate/starch content (Kopper & Lindroth, 2003; Kaakinen *et al.*, 2004), as well as subsequent above-ground and below-ground growth (Isebrands *et al.*, 2001; King *et al.*, 2001, 2005; Karnosky *et al.*, 2003, 2005; Norby *et al.*, 2005; Kubiske *et al.*, 2007).

Physiological data on each of the five different aspen clones grown at the Aspen FACE site have been collected since 1997 (see references in Table 1). One of the most outstanding differences between these clones is the growth enhancement responses resulting from e[CO_2] between clone 216 (0–10% enhancement) and 271 (40–50% enhancement) (Fig. 1). Comparisons of published physiological data have shown that both clones have similar photosynthetic rates, a similar CO_2-induced stimulation in photosynthesis, similar leaf area index (LAI) and a similar increase in LAI in response to e[CO_2], suggesting that the differences in growth stimulation are a result of different strategies in carbon partitioning (Noormets *et al.*, 2001a,b; Karnosky *et al.*, 2005; Riikonen *et al.*, 2008; Taylor *et al.*, 2008). We confirmed that clones 216 and 271 had similar leaf photosynthetic rates at e[CO_2]. Indeed, clone 216 showed a greater stimulation at e[CO_2] (13.66 ± 2.06 µmol m^{-2} s^{-1} in controls vs. 18.76 ± 0.85 µmol m^{-2} s^{-1} in e[CO_2]) than did clone 271 (16.33 ± 0.61 µmol m^{-2} s^{-1} in controls vs. 18.64 ± 0.01 µmol m^{-2} s^{-1} in e[CO_2]) as a result of the lower rates at current [CO_2] (Fig. 2).

Seasonal variation in the response of photosynthesis to e[CO_2] has a large influence on the overwintering capacity of forest trees (Eamus & Ceulemans, 2001). Both clones 216 and 271 showed similar photosynthetic enhancement throughout the season (Noormets *et al.*, 2001b; Riikonen *et al.*, 2008); however, clone 271 showed significant delayed senescence in response to e[CO_2], which extended the growing season by as much as 2 wk compared with 216 (G. Taylor, pers. comm.; Riikonen *et al.*, 2008). Such extension of the growing season is likely to account for *c.* 2–3% of the gross primary productivity of clone 271 (Taylor *et al.*, 2008). Other differences between the clones include minor variations in leaf surface waxes, cytoplasmic lipids, wood starch and nitrogen levels, cell wall thickness, as well as a slight improvement in the capacity of clone 271 to tolerate insects and pathogens (Table 1). Although

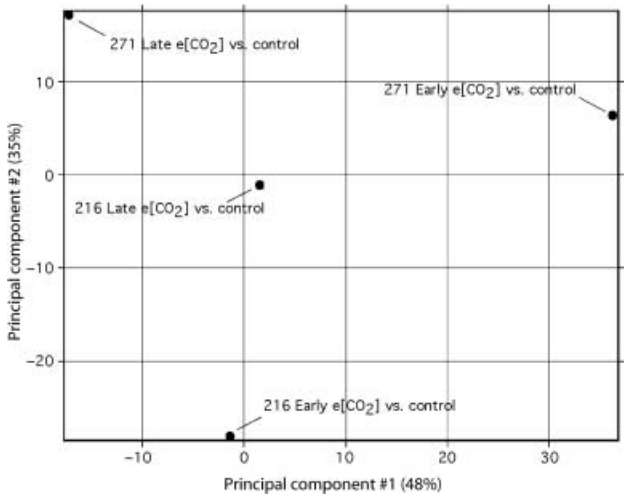

Fig. 3 Principal component analysis (PCA) of normalized transcript intensities with low coefficient of variance (CV < 0.25), showing the principal components explaining the majority of the observed array variation. Principal component #1 correlates with developmental/seasonal differences (early vs. late time points). Principal component #2 correlates with genotypic differences [aspen (*Populus tremuloides*) clone 216 vs. clone 271].

such minor differences do not seem to explain the significant differences between the growth responses of these clones, they do suggest that each clone may use different molecular strategies for carbon accumulation and utilization (Isebrands *et al.*, 2001; Wustman *et al.*, 2001; Karnosky *et al.*, 2003, 2005; Kubiske *et al.*, 2007). As a means to elucidate such molecular differences that may underlie the difference in CO_2 responsiveness between clones 216 and 271, we examined their leaf transcript profiles via microarrays.

Transcriptomics reveals that genotypic variation in transcriptional activity is one of the key factors controlling how clones 216 and 271 respond to e[CO_2]

Microarray analysis of ~26 800 cDNA probes (representing ~10 000 different gene sequences) demonstrated that relatively few leaf transcripts (245) showed a significant response to long-term e[CO_2] in either early (June) or late (August) time points. As visualized by PCA, 83% of the observed variation between leaf transcript expression in clones 216 and 271 can be explained by the interplay of developmental/seasonal differences between the time points and genotypic differences between the clones (Fig. 3). Such interactions between environment and genotype have also been observed in stress and defense responses of aspen genotypes (Osier & Lindroth, 2006).

Comparisons of array data between both clones and time points revealed 184 significantly different ($P < 0.05$) leaf transcripts that responded to long-term e[CO_2] for at least one clone in one of the two time points. Early-season clone 216 showed 52 differentially expressed genes and only 14 genes in the late-season time point. By contrast, early-season clone 271 showed 102 differentially expressed genes and 25 genes in the late-season time point (Tables 2–5). In addition, very few of the leaf e[CO_2]-responsive transcripts overlapped between the datasets for each clone or time point (Fig. 4a). These genotypic and developmental/seasonal differences can also be visualized by HCA, which depicts normalized transcript intensity ratios as coded colors (Fig. 4b). As in PCA, the developmental/seasonal effect was a stronger driver for global gene expression than genotypic difference, leading to the clustering of clone 216 and 271 data by time point. HCA also showed that both the number and magnitude of gene expression changes in response to e[CO_2] were greater earlier in the growing season than late in the season.

Thus, similar to other studies on poplar tree transcriptomic responses to e[CO_2] (Gupta *et al.*, 2005; Taylor *et al.*, 2005; Druart *et al.*, 2006), we observed that field-grown poplar trees exposed to long-term e[CO_2] displayed few significant leaf transcriptional changes compared with trees grown under ambient (control) conditions, despite often significant leaf and stem growth enhancements. The fold changes of the responsive genes in each of these studies were also typically small, making statistically significant detection less likely. Similar small numbers of e[CO_2]-responsive genes and fold changes have been observed in a variety of other plant species (Li *et al.*, 2007), and the most likely explanation is that these studies, like our own, are observing the acclimated response to a chronic treatment rather than the instantaneous response to an acute treatment (Ainsworth *et al.*, 2007).

Clones 216 and 271 showed distinctly different e[CO_2]-responsive sets of leaf transcripts that appeared to differentially impact on multiple biological pathways (Tables 2–5). Many of these gene expression trends showed significant differences in control and experimental expression levels between the clones, often including opposing patterns between the two clones. Such examples are shown in Tables S2–S5 (see Supporting Information), which provide expanded, processed datasets, including spot information, direct comparisons of pair-mean differences between clones and experiments, results of CV analysis and *t*-tests, and log data of both control and experimental samples. Such clonal and opposing trends were also assessed on a sample (~10%) of the genes identified by the microarrays by performing quantitative RT-PCR analyses, using independent RNA samples as a means to more stringently test the patterns (Fig. 5). The combined results highlight the fact that innate transcriptional differences between different tree genotypes are an important and significant factor behind how trees respond to e[CO_2]. This may help to explain some of the variation seen in studies making use of other cDNA microarrays (including the POP1 and POP2 microarrays used in Taylor *et al.*, 2005 and Druart *et al.*, 2006). If genotypes of the same plant species can vary dramatically in their response to e[CO_2], the significant variation observed between different species is not surprising.

Table 2 Differentially regulated genes in leaves of early-season clone 216 grown under elevated [CO_2] (e[CO_2])

Biological function	Definition of best BlastX	Poplar gene model	Fold change	Number of occurrences
Cell rescue defense virulence				
Defense	Thaumatin-like protein [*Arabidopsis thaliana*]	estExt_Genewise1_v1.C_LG_IX1261	–1.37	1
Response to wounding	Wound-induced protein [*Arabidopsis thaliana*]	eugene3.00191063	**–2.25**	1
Energy metabolism				
Electron transport	Thioredoxin [*Glycine max*]	gw1.XVII.864.1	1.56	1
Photosynthesis	ELIP [*Brassica rapa* ssp. *pekinensis*]	eugene3.151410001	1.26	1
Electron transport	Cytochrome *c* oxidase subunit VIa precursor [*Oryza sativa* (japonica cultivar-group)]	estExt_fgenesh4_pg.C_LG_VII1235	–1.45	1
Photosynthesis	PSII DI protein [*Actinidia arguta*]	fgenesh4_pg.C_scaffold_201000016	–1.48	1
Carbon fixation	Ribulose bisphosphate carboxylase [*Gossypium hirsutum*]	eugene3.01230087	–1.51	1
Photosynthesis	Photosystem I reaction center subunit II, chloroplast precursor (PS I subunit 5)	eugene3.00100819	–1.74	1
Photosynthesis	Photosystem II 32 kDa protein [*Riccia huebeneriana*]	gw1.XIII.2252.1	–1.79	1
General metabolism				
Diphosphoinositol	Phosphatidylinositol/phosphatidylcholine transfer protein [*Oryza sativa* (japonica cultivar-group)]	fgenesh4_pm.C_LG_V000599	–1.43	1
Chlorophyll biosynthesis	Glutamate-1-semialdehyde aminotransferase [*Arabidopsis thaliana*]	eugene3.00150799	–1.47	1
Oxidoreductase	Oxidoreductase family protein [*Arabidopsis thaliana*]	estExt_Genewise1_v1.C_410478	–1.51	1
Chlorophyll biosynthesis	Geranylgeranyl hydrogenase [*Glycine max*]	eugene3.00120625	–1.60	1
Hydrolase activity	ATNUDT4 (Nudix hydrolase homolog 4) At1g18300 [*Arabidopsis thaliana*]	grail3.0031000301	–1.61	1
Asparagine synthase	Asparagine synthase (glutamine-hydrolyzing)	eugene3.01070081	**–2.11**	1
Protein fate folding modification				
Proteinase	Aspartic proteinase [*Theobroma cacao*]	estExt_fgenesh4_pg.C_1660009	***2.32***	1
Ubiquitin	Ubiquitin/ribosomal protein CEP52 – wood tobacco	estExt_Genewise1_v1.C_LG_XVI2419	–1.39	1
Transcriptional regulator	Syringolide-induced protein 1-3-1B [*Glycine max*]	estExt_Genewise1_v1.C_LG_I0681	–1.49	1
Proteinase inhibitor	OSJNBb0116K07.15 [*Oryza sativa* (japonica cultivar-group)]	fgenesh4_pg.C_LG_I000354	–1.55	1
Detoxification protein	Copper chaperone [*Populus alba* × *Populus tremula* var. *glandulosa*]	gw1.X.3496.1	–1.59	1
Ubiquitin	Polyubiquitin [*Arabidopsis thaliana*]	eugene3.00060405	–1.85	1
Secondary metabolism				
Cell wall	Peroxidase (EC 1.11.1.7) – upland cotton	grail3.0111002302	***2.55***	**2**
Nitrogen utilization	Phenylcoumaran benzylic ether reductase [*Populus balsamifera* ssp. *trichocarpa*]	estExt_fgenesh4_pm.C_LG_II0164	1.78	1
Biosynthesis	Chalcone synthase (CHS1, clone CHS7)	eugene3.00140920	–1.23	1
Oxidoreductase	Laccase (diphenol oxidase), putative [*Arabidopsis thaliana*]	fgenesh4_pg.C_scaffold_107000055	–1.39	1
Oxidoreductase	Laccase [*Populus trichocarpa*]	estExt_fgenesh4_pg.C_LG_VIII0541	–1.77	1
Protein targeting	Integral membrane protein [*Phaseolus vulgaris*]	estExt_fgenesh4_pg.C_1780025	–1.68	1

Table 2 *continued*

Biological function	Definition of best BlastX	Poplar gene model	Fold change	Number of occurrences
Translation				
Protein biosynthesis	Ribosomal Pr 117 [*Triticum aestivum*]	estExt_Genewise1_v1.C_LG_II4011	–1.24	1
Protein biosynthesis	Ribosomal protein L2 [*Schizanthus pinnatus*]	fgenesh4_pg.C_scaffold_2160000002	–1.66	1
Transport				
Phosphate carrier	Phosphate transporter, mitochondrial	estExt_fgenesh4_pg.C_LG_I2387	–1.40	1
Ion transport	Cyclic nucleotide-gated channel family At5g14870	eugene3.00410125	–1.40	1
Aquaporin	Plasma membrane intrinsic protein [*Populus tremula* × *Populus tremuloides*]	estExt_Genewise1_v1.C_LG_III0271	–1.75	1
Xanthine/uracil	Xanthine/uracil permease family protein At2g26510 [*Arabidopsis thaliana*]	gw1.40.581.1	–1.76	1
Aquaporin	Aquaporin [*Ricinus communis*]	eugene3.00280238	–1.87	1
Unclassified				
Phosphate carrier	Predicted P0524G08.101 gene product [*Oryza sativa* (japonica cultivar-group)]	gw1.XIII.3248.1	–1.27	1
Mitochondrion	Mitochondrial protein of unknown function, overexpression suppresses an rpo41 mutation affecting mitochondrial RNA polymerase	gw1.XIII.1305.1	–1.33	1
Unknown	Hypothetical protein Npun02000359 [*Nostoc punctiforme* PCC 73102]	fgenesh4_pg.C_scaffold_14076000001	–1.35	**2**
Cell wall	Projectin	eugene3.00100655	–1.36	1
Mitochondrion	Sterility protein 1 [*Phaseolus vulgaris*]	eugene3.02970009	–1.48	1
Leucine-rich repeat	HcrVf2 protein [*Malus floribunda*]	eugene3.00101000	–1.51	1
Protein phosphorylation	Protein kinase, putative	fgenesh4_pg.C_LG_XI001073	–1.57	1
Unknown	Unknown	grail3.0068005901	–1.58	1
Unknown	Unknown	eugene3.186940001	–1.60	1
Zinc-finger	Unknown	eugene3.02270010	–1.60	**10**
Conserved protein Mo25	AT5g47540/MNJ7_13 [*Arabidopsis thaliana*]	estExt_fgenesh4_pg.C_LG_VI0133	–1.61	1
Unknown	Unknown	gw1.XVI.2246.1	–1.62	1
PSI assembly	ycf3 protein [*Panax ginseng*]	estExt_Genewise1_v1.C_14480001	–1.68	1
Unknown	Expressed protein [*Arabidopsis thaliana*]	estExt_fgenesh4_pg.C_LG_XIV0052	–1.72	1
Unknown	Unknown protein [*Oryza sativa* (japonica cultivar-group)]	estExt_fgenesh4_pg.C_LG_IX1163	–1.77	1
Protein phosphorylation	Unknown protein [*Arabidopsis thaliana*]	estExt_fgenesh4_pm.C_LG_XI0040	–1.78	1
Zinc-binding	Zinc-binding protein [*Oryza sativa* (japonica cultivar-group)]	grail3.0031020101	–1.99	1

Functional categories were based on the biological/molecular functions and standardized KOGG descriptions from the Joint Genome Initiative (JGI) gene ontology within the poplar database. Bold and italic type in the fold change column indicates two-fold or greater up-regulation, and bold type indicates two-fold or greater down-regulation.

Table 3 Differentially regulated genes in leaves of early-season clone 271 grown under elevated [CO_2] (e[CO_2])

Biological function	Definition of best BlastX	Poplar gene model	Fold change	Number of occurrences
Cell fate				
Senescence	Senescence-associated protein [*Pisum sativum*]	gw1.7267.9.1	–1.71	1
Cell rescue defense virulence				
Defense, membrane permeability	Thaumatin/osmotin [*Arabidopsis thaliana*]	gw1.I.8918.1	***6.72***	1
Nutrient reservoir	Germin-like protein [*Arabidopsis thaliana*]	fgenesh4_pg.C_scaffold_426000001	***5.53***	1
Proteinase inhibitor	Kunitz trypsin inhibitor 3 [*Populus balsamifera* ssp. *trichocarpa* × *Populus deltoides*]	estExt_fgenesh4_pg.C_LG_XIX0984	***3.00***	***5***
Proteinase inhibitor	Kunitz trypsin inhibitor TI3 [*Populus tremula*]	estExt_Genewise1_v1.C_14340008	***2.29***	1
Defense	Thaumatin-like protein [*Actinidia deliciosa*]	grail3.0020019002	1.85	1
Defense	Thaumatin-like protein [*Arabidopsis thaliana*]	estExt_Genewise1_v1.C_LG_IX1261	–1.67	1
Glutathione metabolism	Glutathione *S*-transferase GST 23 [*Glycine max*]	estExt_fgenesh4_pg.C_LG_III1026	–1.82	1
Response to wounding	Protease inhibitor 2 [*Zinnia elegans*]	eugene3.10700003	**–2.29**	1
Transcription	DREB-like protein [*Cucumis melo*]	gw1.XVIII.1182.1	**–2.69**	1
Cellular communication				
Protein transport	GTP-binding protein [*Oryza sativa* (japonica cultivar-group)]	estExt_fgenesh4_pm.C_LG_I0698	–1.65	1
Transcriptional regulation	Dentin sialophosphoprotein preproprotein [*Homo sapiens*]	gw1.XV.2357.1	–1.72	1
Development systemic				
Meristem development	ASYMMETRIC LEAVES2-like gene 1 protein [*Arabidopsis thaliana*]	gw1.V.3873.1	1.82	1
Transcription	MADS-box protein PTM5 [*Populus tremuloides*]	gw1.XIV.941.1	–1.58	1
Energy metabolism				
Reductase	1,4-Benzoquinone reductase [*Oryza sativa* (japonica cultivar-group)]	estExt_Genewise1_v1.C_LG_XI2337	1.79	1
Glycolysis	Enolase, isoform 1 [*Hevea brasiliensis*]	estExt_fgenesh4_pm.C_280132	1.44	1
Photosynthesis	Photosystem I reaction center subunit II, chloroplast precursor (PS I subunit 5)	eugene3.00081422	–1.30	1
Chloroplast	Oxygen-3volving enhancer protein 1	estExt_fgenesh4_kg.C_LG_VII0034	–1.39	1
Photosynthesis	Ribulose-1,5-bisphosphate carboxylase/oxygenase activase 1 [*Gossypium hirsutum*]	gw1.VIII.2613.1	–1.43	**2**
Putative thioredoxin	Unknown [*Arabidopsis thaliana*]	estExt_Genewise1_v1.C_LG_XIII1459	–1.44	1
Photosynthesis	Chlorophyll *a*/*b*-binding protein precursor [*Euphorbia esula*]	grail3.0002067901	–1.44	**3**
Photosynthesis	Ultraviolet-B-repressible protein [*Gossypium hirsutum*]	estExt_Genewise1_v1.C_280006	–1.46	1
Photosynthesis	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [*Trithrinax acanthocoma*]	gw1.211.2.1	–1.64	1
Electron transport	Blue copper protein [*Pisum sativum*]	estExt_fgenesh4_pg.C_LG_II0928	–1.99	1
Phosphatidylcholine metabolism	Phospholipase D [*Ricinus communis*]	fgenesh4_pm.C_scaffold_44000016	**–2.02**	1
General metabolism				
Amino acid transport	Amino acid carrier [*Ricinus communis*]	eugene3.06730001	***12.29***	1
Nitrogen metabolism	Aminomethyltransferase precursor [*Mesembryanthemum crystallinum*]	estExt_fgenesh4_pm.C_LG_XI0012	–1.35	1
Spermidine biosynthesis	*S*-Adenosylmethionine decarboxylase [*Prunus persica*]	gw1.123.91.1	–1.51	1
Fatty acid biosynthesis	Acetyl-CoA carboxylase β-subunit [*Ipomoea batatas*]	estExt_Genewise1_v1.C_1330169	–1.53	1
UDP-glucosyl transferase	Unknown	fgenesh4_pg.C_LG_I000366	–1.57	1
Tyrosine biosynthesis	Unknown	gw1.97.33.1	–1.68	1
Pectin metabolism	Unknown	eugene3.00290334	–1.70	1
Hydrolase, putative	Hypothetical protein T1K7.26 – *Arabidopsis thaliana*	grail3.0010026002	–1.71	1
Transferase	Glycosyltransferase [*Gossypium raimondii*]	estExt_Genewise1_v1.C_LG_V4069	–1.73	1
Electron transport	P450 monooxygenase [*Gossypium arboreum*]	eugene3.01270031	–1.78	1

Table 3 *continued*

Biological function	Definition of best BlastX	Poplar gene model	Fold change	Number of occurrences
Phosphatidylserine biosynthesis	Phosphatidylserine sythase family protein	eugene3.00081172	–1.83	1
Cell wall	Pectin methylesterase [*Populus tremula* × *Populus tremuloides*]	grail3.0150001701	–1.87	1
Methionine biosynthesis	Cobalamine-independent methionine synthase [*Solenostemon scutellarioides*]	grail3.0066005802	–1.91	1
Electron transport	Respiratory burst oxidase homolog [*Nicotiana benthamiana*]	estExt_Genewise1_v1.C_LG_III1154	–1.99	1
Response to pests	Chitinase-like protein [*Gossypium hirsutum*]	estExt_Genewise1_v1.C_LG_X0543	**–2.11**	1
Protein fate folding modification				
Protein folding	Cyclophilin [*Populus alba* × *Populus tremula*]	estExt_fgenesh4_pg.C_LG_IX0365	–1.51	1
Ubiquitin	Ubiquitin-conjugating enzyme-like protein [*Oryza sativa* (japonica cultivar-group)]	gw1.107.100.1	–1.55	1
Ubiquitin	Ubiquitin/ribosomal protein CEP52 – wood tobacco	estExt_Genewise1_v1.C_LG_XVI2419	–1.58	1
Endopeptidase	At1g67700/F12A21_30 [*Arabidopsis thaliana*] contains domain OLIGOPEPTIDASE	estExt_Genewise1_v1.C_LG_X4972	–1.78	1
Regulation interaction with cellular environment				
Light-inducible	Light-inducible protein ATLS1 [*Arabidopsis thaliana*]	grail3.0023033701	–1.12	1
Na+/H+ antiporter, putative	Sodium hydrogen antiporter [*Arabidopsis thaliana*]	eugene3.00012381	–1.83	1
Secondary metabolism				
Oxidoreductase	Laccase [*Arabidopsis thaliana*]	eugene3.00060812	–1.60	1
Cell wall	Peroxidase [*Populus nigra*]	estExt_fgenesh4_pm.C_LG_I0036	–1.67	1
Lignin biosynthesis	Cinnamoyl-CoA reductase [*Arabidopsis thaliana*]	gw1.II.2127.1	–1.76	1
Transferring hexosyl groups	UDP-glucuronosyltransferase [*Arabidopsis thaliana*]	gw1.II.1756.1	–1.87	1
Subcellular localization				
Endopeptidase	Signal peptide peptidase [*Galega orientalis*]	estExt_fgenesh4_pm.C_LG_IX0436	–1.70	1
Cell adhesion, cell wall	Fasciclin-like AGP 4 [*Populus alba* × *Populus tremula*]	eugene3.00131210	–1.82	1
Cell adhesion, cell wall	Fasciclin-like AGP 5 [*Populus alba* × *Populus tremula*]	grail3.0094006801	**–2.05**	1
Transcription				
	Ankyrin repeat-like protein [*Oryza sativa* (japonica cultivar-group)]	fgenesh4_pg.C_LG_VIII001583	1.74	1
Protein biosynthesis	Remorin family protein [*Arabidopsis thaliana*]	estExt_Genewise1_v1.C_LG_XII1403	–1.44	1
Transcription	Apetala2/ethylene responsive factor [*Populus alba* × *Populus tremula*]	gw1.158.150.1	–1.46	1
Putative polyadenylation	FY protein [*Oryza sativa* (japonica cultivar-group)]	gw1.III.2677.1	–1.56	1
Nucleic acid binding	Spliceosomal protein FBP21	estExt_fgenesh4_pg.C_LG_IX0581	–1.68	1
Transcription	Ethylene-responsive factor-like protein 1 [*Capsicum annuum*]	gw1.XIV.2445.1	–1.72	1
myb transcription factor	Transcription factor [*Arabidopsis thaliana*] member of the R2R3 factor gene family	fgenesh4_pg.C_LG_VII000767	–1.84	1
Translation				
Protein biosynthesis	Translation initiation factor B04 [*Helianthus annuus*]	estExt_Genewise1_v1.C_LG_X0725	–1.27	1
Protein biosynthesis	Ribosomal protein L17 [*Oryza sativa* (japonica cultivar-group)]	estExt_Genewise1_v1.C_LG_XV2068	–1.35	1
Protein biosynthesis	Acidic ribosomal protein P1a	eugene3.00021666	–1.47	1
tRNA synthetase	tRNA synthetase class I (C) family protein [*Arabidopsis thaliana*]	estExt_fgenesh4_pm.C_LG_X0474	–1.50	1
Protein biosynthesis	40S ribosomal protein, putative	estExt_fgenesh4_pm.C_LG_VI0643	–1.56	1
Protein biosynthesis	40S ribosomal protein S8	estExt_Genewise1_v1.C_LG_IX3421	–1.66	1
Transport				
Nitrate transporter	Nitrate transporter [*Populus tremula* × *Populus tremuloides*]	fgenesh4_pm.C_LG_IX000714	***2.11***	1
Vesicle coat protein	OSJNBb0017I01.5 [*Oryza sativa* (japonica cultivar-group)]	estExt_Genewise1_v1.C_1240035	–1.63	1
Vesicle transport	Synaptobrevin [*Arabidopsis thaliana*]	gw1.I.7543.1	–1.68	1
Phosphate carrier	Plastidic phosphate translocator-like protein2 [*Mesembryanthemum crystallinum*]	estExt_fgenesh4_pm.C_LG_I0645	–1.68	1
Protein transport	Longevity-assurance (LAG1) family protein At1g13580/F13B4_25 [*Arabidopsis thaliana*]	gw1.VIII.2932.1	–1.79	1

Table 3 *continued*

Biological function	Definition of best BlastX	Poplar gene model	Fold change	Number of occurrences
Unclassified				
GTP-ase	Unknown	eugene3.00090816	1.87	1
Unknown	Unknown	eugene3.00011774	1.62	1
Protein phosphorylation	Choline kinase [*Arabidopsis thaliana*]	grail3.0028024201	1.59	1
Metabolism	No hit found	eugene3.01500033	1.37	1
Arabidopsis protein of unknown function DUF266	At5g11730 [*Arabidopsis thaliana*]	estExt_fgenesh4_pm.C_LG_VI0698	1.34	1
Unknown	Unknown	eugene3.00191070	–1.27	1
Unknown	Unknown	eugene3.01350001	–1.31	1
Glycolipid transfer	Glycolipid transfer protein-like [*Oryza sativa* (japonica cultivar-group)]	eugene3.00100340	–1.36	1
Unknown	Unknown	eugene3.00120229	–1.41	1
Unknown	TO71-3 [*Taraxacum officinale*]	grail3.11474000101	–1.49	1
Phosphate carrier	PREDICTED P0524G08.101 gene product [*Oryza sativa* (japonica cultivar-group)]	gw1.XIII.3248.1	–1.52	1
Unknown	At3g60850 [*Arabidopsis thaliana*]	eugene3.00140158	–1.54	1
Unknown	Hypothetical protein [*Arabidopsis thaliana*]	estExt_Genewise1_v1.C_LG_XIV1980	–1.56	1
Membrane protein	Integral membrane family protein-like [*Oryza sativa* (japonica cultivar-group)]	estExt_fgenesh4_pm.C_LG_X0826	–1.58	1
GTP binding	NTGP4 [*Nicotiana tabacum*]	gw1.41.462.1	–1.59	1
Unknown	Unknown protein [*Oryza sativa* (japonica cultivar-group)]	estExt_Genewise1_v1.C_95310004	–1.60	1
Proteinase	OTU-like cysteine protease-like [*Oryza sativa* (japonica cultivar-group)]	gw1.XIV.2913.1	–1.62	**2**
Unknown	Unknown	eugene3.00102359	–1.63	1
Unknown	NtEIG-E80 [*Nicotiana tabacum*]	gw1.XVI.3414.1	–1.64	1
Unknown	Unknown [*Arabidopsis thaliana*]	eugene3.00040773	–1.64	1
Unknown	OJ991214_12.11 [*Oryza sativa* (japonica cultivar-group)]	grail3.0010069001	–1.69	1
Protein phosphorylation	Unknown	grail3.0140005901	–1.70	1
Chlorophyll metabolism	Aminotransferase [*Arabidopsis thaliana*]	fgenesh4_pm.C_LG_V000349	–1.73	1
Unknown	No hit found	grail3.1043000101	–1.73	1
Calcium binding	Unknown [*Arabidopsis thaliana*]	grail3.0242000102	–1.73	1
Protein dephosphorylation	AT4g31860/F11C18_60 [*Arabidopsis thaliana*]	eugene3.00061958	–1.75	1
Protein phosphorylation	No hit found	gw1.29.518.1	–1.95	1
Zinc-finger	NEW1 domain containing protein isoform [*Oryza sativa* (japonica cultivar-group)]	gw1.VII.2281.1	–1.99	1
Protein phosphorylation	Secreted glycoprotein 3	eugene3.01340020	**–2.17**	1
Carbohydrate metabolism	No hit found	eugene3.00090917	**–2.55**	1
Structural	At5g09820 [*Arabidopsis thaliana*]	gw1.I.3658.1	**–2.62**	1

Functional categories were based on the biological/molecular functions and standardized KOGG descriptions from the Joint Genome Initiative (JGI) gene ontology within the poplar database. Bold and italic type in the fold change column indicates two-fold or greater up-regulation, and bold type indicates two-fold or greater down-regulation.

Table 4 Differentially regulated genes in leaves of late-season clone 216 grown under elevated [CO_2] (e[CO_2])

Biological function	Definition of best BlastX	Poplar gene model	Fold change	Number of occurrences
Cellular communication				
Receptor kinase	Receptor kinase-like protein [*Oryza longistaminata*]	grail3.0067011801	1.20	1
Development systemic				
Meristem maintenance	NAC domain protein NAC4 [*Glycine max*]	fgenesh4_pg.C_scaffold_107000083	***6.91***	1
Energy metabolism				
Ion transport	ATPase β-subunit [*Nicotiana sylvestris*]	estExt_Genewise1_v1.C_LG_VIII1306	–1.28	1
Photosynthesis	Chlorophyll *a*/*b*-binding protein precursor – upland cotton chloroplast	grail3.0002067901	–1.38	1
Photosynthesis	Oxygen-evolving enhancer protein 1 precursor [*Bruguiera gymnorrhiza*]	estExt_fgenesh4_kg.C_LG_VII0034	–1.41	1
Iron metabolism	Thioredoxin [*Arabidopsis thaliana*]	estExt_Genewise1_v1.C_LG_XIII2097	–1.43	1
General metabolism				
Cell wall biosynthesis	UDP-sugar pyrophosphorylase [*Pisum sativum*]	eugene3.00020715	***2.39***	1
Metabolism	Cytochrome P450 monooxygenase – maize (fragment)	estExt_Genewise1_v1.C_91780006	1.18	1
Protein fate folding modification				
Ubiquitin	Ubiquitin-specific protease 26 [*Arabidopsis thaliana*]	estExt_fgenesh4_pg.C_LG_II0732	1.70	1
Storage				
Cell wall	Acid phosphatase class B family protein At1g04040/F21M11_2 [*Arabidopsis thaliana*]	estExt_Genewise1_v1.C_LG_II4012	1.33	1
Systemic regulation of/interaction with environment				
Response to stress	Iron transport protein 2 [*Ricinus communis*]	eugene3.00130613	***2.46***	1
Unclassified				
Unknown	Unknown protein [*Arabidopsis thaliana*]	gw1.IX.4902.1	***2.70***	1
Unknown	Unknown	eugene3.00051241	–1.28	1
Protein phosphorylation	Mitochondrial protein of unknown function, overexpression suppresses an rpo41 mutation affecting mitochondrial RNA polymerase	fgenesh4_pg.C_LG_XII001221	–1.67	1

Functional categories were based on the biological/molecular functions and standardized KOGG descriptions from the Joint Genome Initiative (JGI) gene ontology within the poplar database. Bold and italic type in the fold change column indicates two-fold or greater up-regulation, and bold type indicates two-fold or greater down-regulation.

Table 5 Differentially regulated genes in leaves of late-season clone 271 grown under elevated [CO_2] (e[CO_2])

Biological function	Definition of best BlastX	Poplar gene model	Fold change	Number of occurrences
Cell cycling processing				
Chromosome assembly	Regulator of chromosome condensation (RCC1) family protein [*Arabidopsis thaliana*]	eugene3.01230071	1.14	1
Cell rescue defense virulence				
Ion binding	Metallothionein 2b [*Populus trichocarpa* × *Populus deltoides*]	eugene3.00091335	1.39	**17**
Ion binding	Metallothionein 1a [*Populus balsamifera* ssp. *trichocarpa* × *Populus deltoides*]	estExt_fgenesh4_pg.C_2080003	1.36	**4**
Ion binding	Metallothionein 1b [*Populus balsamifera* ssp. *trichocarpa* × *Populus deltoides*]	estExt_Genewise1_v1.C_1420036	1.33	**14**
Ion binding	Metallothionein 1a [*Populus balsamifera* ssp. *trichocarpa* × *Populus deltoides*]	eugene3.01200081	1.32	**10**
Proteinase inhibitor	Kunitz trypsin inhibitor 3 [*Populus balsamifera* ssp. *trichocarpa* × *Populus deltoides*]	estExt_fgenesh4_pg.C_LG_XIX0984	**–2.58**	**2**
Cellular organization				
Tubulin binding	Tubulin folding cofactor D [*Arabidopsis thaliana*]	gw1.XIV.724.1	1.45	1
Actin depolymerizing	Actin-depolymerizing factor 2 [*Petunia* × *hybrida*]	estExt_fgenesh4_pg.C_LG_I1779	–1.56	1
Protein fate folding modification				
Unknown	31.2-kDa small heat shock family protein/hsp20 family protein [*Arabidopsis thaliana*]	eugene3.00140883	1.40	1
Proteinase inhibitor	Cystatin-like protein [*Citrus* × *paradisi*]	fgenesh4_pg.C_LG_VI000133	–1.47	1
Secondary metabolism				
Transferase	AER [*Nicotiana tabacum*]	eugene3.00161069	1.21	1
Spermidine biosynthesis	Arginine decarboxylase 1 [*Datura stramonium*]	estExt_Genewise1_v1.C_LG_IV0804	–1.86	1
Subcellular localization				
Chromosome assembly	Histone H3.2 protein [*Oryza sativa* (japonica cultivar-group)]	estExt_Genewise1_v1.C_LG_II0922	1.30	1
Transcription				
DNA binding	myb family transcription factor At4g36570 [*Arabidopsis thaliana*]	gw1.VII.3578.1	1.81	1
Protein catabolism	Protein involved in mRNA stability	gw1.VIII.145.1	1.23	1
Histone modification	TAZ zinc finger family protein/zinc finger (type) family protein [*Arabidopsis thaliana*]	estExt_fgenesh4_pg.C_1350050	–1.22	1
Transcription	Transcription factor of the Forkhead/HNF3 family	estExt_Genewise1_v1.C_LG_II4045	–1.32	1
Translation				
Protein biosynthesis	Translation initiation factor B04 [*Helianthus annuus*]	estExt_Genewise1_v1.C_LG_X0725	1.24	1
Transport				
Aquaporin	Plasma membrane intrinsic protein [*Populus tremula* × *Populus tremuloides*]	grail3.0049030302	1.35	1
Unclassified				
Unknown	Unknown	gw1.XVI.2635.1	***2.47***	1
Unknown	Protein [*Arabidopsis thaliana*]	eugene3.00120098	1.82	1
Unknown	Hypothetical chloroplast RF2 [*Eucalyptus globulus* ssp. *globulus*]	eugene3.110670001	1.41	1
Unknown	Unknown	grail3.0006039501	1.26	1
Unknown	Similar to unknown protein [*Arabidopsis thaliana*]	fgenesh4_pg.C_LG_X001237	1.16	1
Membrane protein	Unknown	grail3.0012030401	–1.61	1

Functional categories were based on the biological/molecular functions and standardized KOGG descriptions from the Joint Genome Initiative (JGI) gene ontology within the poplar database. Bold and italic type in the fold change column indicates two-fold or greater up-regulation, and bold type indicates two-fold or greater down-regulation.

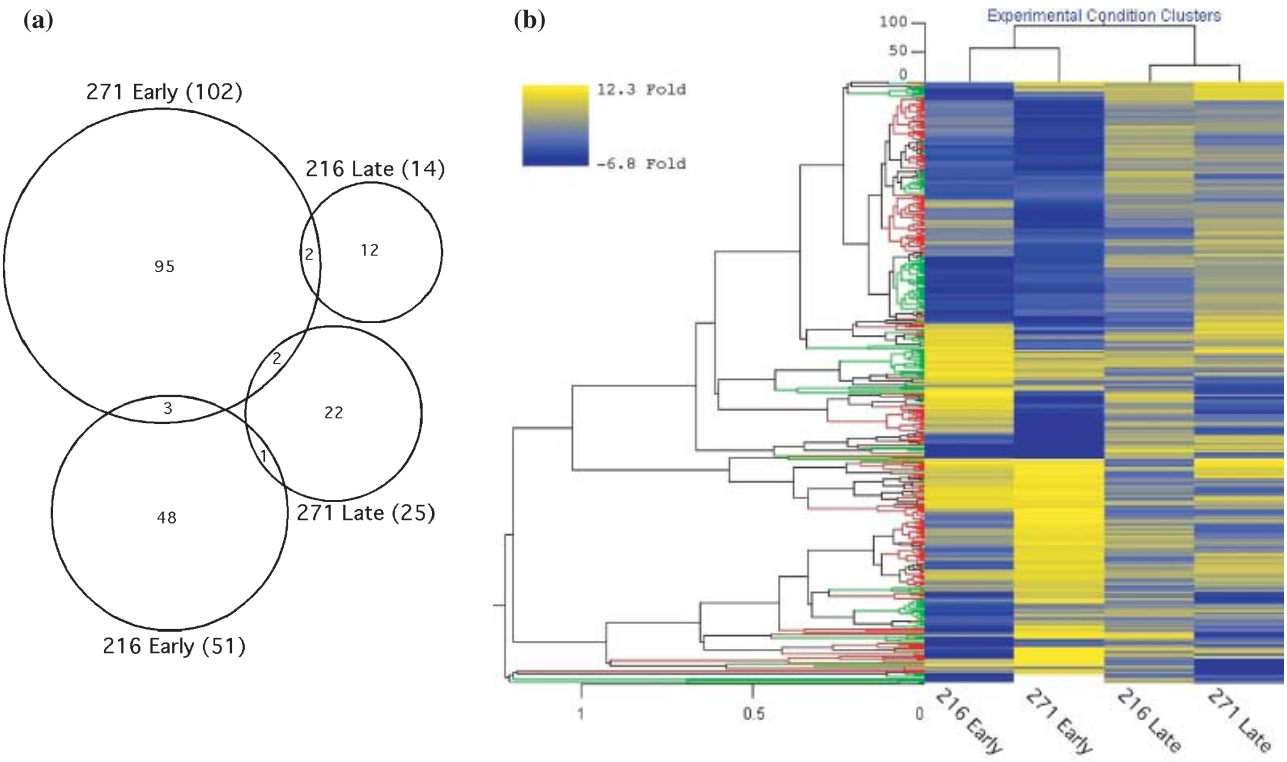

Fig 4 (a) Venn diagram comparing the differently expressed transcripts that were statistically significant ($P < 0.05$) for each aspen (*Populus tremuloides*) clone and time point. The analysis indicates that there is little overlap between the statistically significant sets of transcripts that are differentially expressed in response to long-term elevated [CO_2] (e[CO_2]). (b) Hierarchical cluster analysis (HCA) of normalized transcript intensity ratios having statistical significance ($P < 0.05$) in at least one condition for early and late time points for clones 216 and 271. Yellow indicates up-regulation in response to e[CO_2] compared with control. Blue indicates down-regulation compared with control. Gray indicates little change compared with control. Stronger changes occur in response to e[CO_2] earlier in the growing season than late in the season. Red branches indicate data that show statistically significant differential expression between the control and e[CO_2] samples for early-season clone 271. Green branches indicate data that are not statistically significant for early-season clone 271.

It should be noted that there are some limitations to the PICME arrays used in this study in that they are not fully comprehensive and contain a moderate level of gene redundancy. It may be argued that, because these arrays contain ~3600 leaf/shoot ESTs, ~9000 xylem/cambium ESTs and ~8600 root ESTs, they cannot be effectively used for leaf transcriptomics. However, leaves also contain xylem tissue, and many of the represented genes/ESTs are expressed in many different tissues. For example, of the leaf expression patterns assessed by quantitative RT-PCR (Fig. 5), only seven of these sequences were originally identified in leaf/shoot EST libraries. The remainder were identified as ESTs in other tissues (six from xylem/cambium and eight from root EST libraries), yet they are clearly expressed in leaves. There are many similar examples throughout our microarray data, as well as the data of leaf transcriptomics studies using the PICME arrays and/or similar POP1 and POP2 cDNA arrays (Brosché *et al.*, 2005; Taylor *et al.*, 2005; Druart *et al.*, 2006; Rinaldi *et al.*, 2007; Fluch *et al.*, 2008). We also view the redundancy of the PICME arrays as an advantage, as it provides additional hybridization signals for each gene. In this respect, the transcriptional patterns of redundant clones on the PICME arrays corresponded well. Examples can be found in Tables 2–5, as well as the tables in the Supporting Information.

Clones 216 and 271 respond to e[CO_2] through fundamentally different biological strategies that impact multiple pathways

As a means to highlight the primary (most significant) leaf gene expression trends observed between clones 216 and 271 as they sense and respond to e[CO_2], we grouped the e[CO_2]-responsive genes into functional categories based on the biological/molecular functions and standardized KOGG descriptions from the JGI gene ontology within the poplar database. Here, we discuss the key functional categories independently.

Cell rescue and defense Clone 271 appears to have a different approach to defense compared with clone 216. Although early-season clone 216 shows a slight decrease in the expression of some leaf cell rescue and defense genes (Table 2),

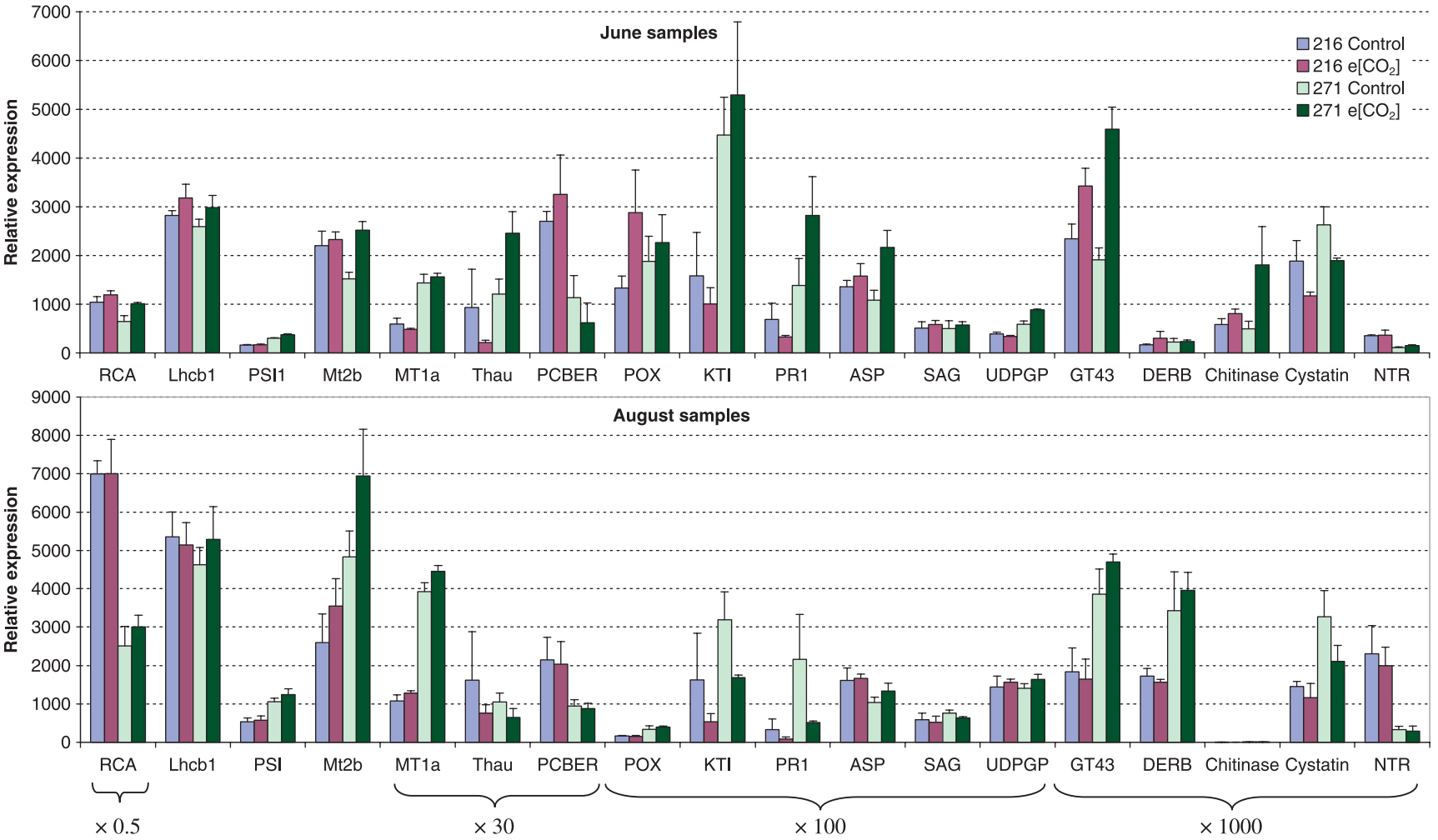

Fig. 5 Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) comparisons of selected genes between aspen (*Populus tremuloides*) clones 216 and 271 in response to long-term elevated [CO_2] (e[CO_2]). Included genes are as follows: RCA, ribulose-1,5-bisphosphate carboxylase/oxygenase activase; Lhcb1, chlorophyll *a*/*b*-binding protein; PSI, photosystem I reaction center subunit II; MT2b, metallothionein 2b; MT1, metallothionein 1; Thau, thaumatin; PCBER, phenylcoumaran benzylic ether reductase; POX, peroxidase; KTI, kunitz trypsin inhibitor 3; PR1, pathogenesis-related protein; ASP, aspartic proteinase; SAG, senescence-associated protein; UDPGP, UDP-sugar pyrophosphorylase; GT43, glycosyl transferase; DREB, DREB-like transcription factor; chitinase; cystatin; NTR, nitrate transporter.

early-season clone 271 demonstrates the up-regulation of members of this gene class, including the up-regulation of thaumatin (Thau, 6.72-fold), germin-like protein (5.53-fold), pathogenesis-related protein (PR1), chitinase and repeated occurrences of kunitz trypsin inhibitors (KTI3, ~3.00-fold) (Table 3; Fig. 5). Indeed, at both early and late time points, clone 216 has either significantly reduced or opposing trends in the expression of such genes compared with clone 271. In the late-season samples, these trends reverse for clone 271 (showing down-regulation in response to e[CO_2]), whereas the expression levels and responses remain approximately the same in clone 216 (Tables 4, 5; Fig. 5). In addition, late-season clone 271 differential gene expression is dominated by a slight up-regulation in metallothionein genes (MT1a and MT2b), which are expressed at much higher levels than by clone 216 (Table 5). Metallothionein genes are also involved in cell defense and stress responses through their ability to bind metal ions. Likewise, there was a significant up-regulation of a dehydration-responsive element-binding (DREB)-like transcription factor in late-season clone 271 compared with clone 216 (Fig. 5). DREB transcription factors have been shown to regulate the expression of other stress-related genes in the biotic stress signaling pathway (Agarwal *et al.*, 2006). In addition, it has been possible to engineer stress tolerance in transgenic plants by manipulating the expression of DREBs (Agarwal *et al.*, 2006), and so this may be one gene that helps clone 271 deal with stress late in the growing season.

Such observations indicate that the strategies for defense and leaf stress responses between clones 216 and 271 are different, and that clone 271 seems to take action early in the season to protect itself against attack and/or other stresses, whereas clone 216 directs this energy elsewhere. This correlates well with the fact that clone 271 shows an improved ability to deal with rust infections in the leaves and tent caterpillar attack compared with clone 216 (Kopper & Lindroth, 2003; Mankovska *et al.*, 2005; Osier & Lindroth, 2006). Likewise, clone 271 appears to adapt to late-season stresses largely through altered transcriptional activity, whereas clone 216 does not.

Photosynthesis It is interesting to note that, despite the well-characterized e[CO_2]-induced increases in photosynthesis (Table 1; Fig. 2), the microarray data indicated a slight (< 1.75-fold) down-regulation of leaf genes related to photosynthesis and energy metabolism (Tables 2, 3). This phenomenon has also been observed in other projects using *Populus* species to characterize the effects of e[CO_2], employing similar EST microarrays as used in our study (Taylor *et al.*, 2005). Quantitative RT-PCR analysis indicated an e[CO_2]-induced trend in photosynthesis, including Rubisco activase (RCA), chlorophyll *a*/*b*-binding protein (Lhcb1) and photosystem I reaction center subunit II (PSI). However, significant expression differences were only observed as a result of genotypic or seasonal variations, rather than those triggered by e[CO_2] (Fig. 5). Such minor differences between microarray and quantitative RT-PCR results are probably caused by some level of cross-hybridization on the cDNA arrays, and are not uncommon when assessing genes having small fold-changes. The primers designed for quantitative RT-PCR are often more gene or group specific, and can thus discriminate desired expression from that of related genes. Overall, the expression trends (relative increases and decreases) for the quantitative RT-PCR results are consistent with the arrays, especially when comparing trends between clones 216 and 271.

Translational-, post-translational- and metabolism-level adjustments are also key to long-term physiological and growth responses in these trees. For example, in terms of photosynthesis, there was a substantial increase in the levels of RCA transcripts in clone 216 at the late time point compared with clone 271 (Fig. 5). RCA controls the overall activity of photosynthesis by post-translationally regulating the activity of the Rubisco enzyme through the removal of ribulose 1,5-bisphosphate (RuBP) from inactive Rubisco. Likewise, changes in the activity of Rubisco are related to alterations in source–sink relationships that may be controlled by limitations in the supply of nitrogen and/or water (Long *et al.*, 2004; Ainsworth & Rogers, 2007; Luo *et al.*, 2008). This correlates with changes in the expression levels of nitrogen utilization genes (see below) and water transport genes, including aquaporin/plasma membrane intrinsic protein (Tables 2, 5). Reduced leaf transcript abundances of aquaporins in response to e[CO_2] were also observed for clone 216 by Gupta *et al.*, (2005) and, combined with observed reductions in stomatal conductance (Table 1), may suggest that e[CO_2]-treated trees are able to manage water more efficiently (Tjoelker *et al.*, 1998).

Primary sugar metabolism There was no effect of e[CO_2] on the levels of glucose, fructose or sucrose, and only a marginally significant increase in starch ($P = 0.094$) for each clone (Fig. 6). However, there were significantly higher glucose ($P = 0.004$), fructose ($P = 0.015$), sucrose ($P = 0.013$) and starch ($P = 0.022$) contents in clone 271 compared with clone 216. The effect of e[CO_2] on starch content was more pronounced in clone 271, as indicated by the significant $CO_2 \times$ clone interaction, and this suggests that clone 271 may partition more carbon into transitory leaf starch at the beginning of the season to provide a greater carbon pool for nocturnal metabolism. The high sucrose levels in clone 271 could be indicative of a high flux of sucrose into the phloem, as well as a high capacity for carbon utilization. Lower levels of sucrose in clone 216 might indicate that more carbon is being funneled into leaf secondary metabolism rather than export.

Changes in gene expression related to leaf carbohydrate metabolism were also observed in the array data. Increases in a cell wall-associated glycosyl transferase (GT43) were seen in both clones. Glycosyltransferases are enzymes that act as catalysts for the transfer of monosaccharide units from an activated sugar phosphate to an acceptor molecule. Likewise, UDP-sugar pyrophosphorylase (UDPGP) is involved in

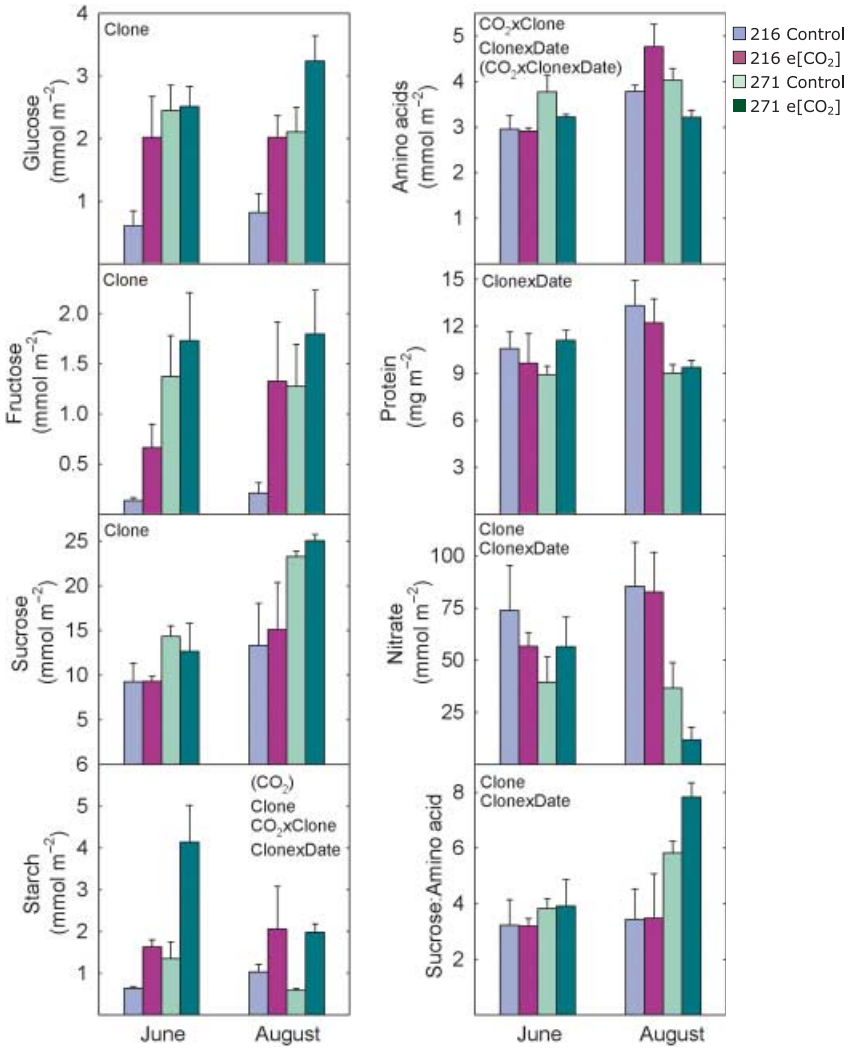

Fig. 6 Levels of carbon and nitrogen metabolites in the leaves of aspen (*Populus tremuloides*) clones 216 and 271 grown at current [CO_2] and long-term elevated [CO_2] (e[CO_2]). Leaves were taken from the same upper canopy collection as those used in the array and quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analyses. Significant ($P < 0.05$) effects of CO_2, clone and date and the interactions (CO_2 × clone, clone × date, CO_2 × clone × date) are indicated in the panels. Marginally significant ($P < 0.1$) effects are indicated by parentheses surrounding the treatment.

general metabolism by controlling the levels of the internal hexose phosphate pool by providing metabolites for both cell wall synthesis and starch synthesis. The observed increase in expression (2.39-fold) of this sugar pyrophosphorylase in late-season clone 216 suggests that glucose and fructose may be directed towards cell wall thickening in the leaves or possibly the stems of clone 216 (Table 4). We consider this possibility next.

Secondary metabolism This study reveals that secondary metabolism genes, such as phenylcoumaran benzylic ether reductase (PCBER) and cell wall peroxidase (POX), are more strongly up-regulated in the leaves of clone 216 at the early time point (1.78-fold and 2.55-fold, respectively) in response to e[CO_2] (Tables 2, 3; Fig. 5). Opposite trends in PCBER between the clones also suggest opposing activity of the phenylpropanoid pathway between clones 216 and 271 in response to e[CO_2]. In addition, clone 271 exhibits the down-regulation of genes involved in leaf secondary metabolism, including the lignin biosynthesis genes cinnamoyl-CoA reductase (−1.76-fold) and laccase (−1.60-fold). There is a corresponding down-regulation of leaf cell wall-related genes in clone 271, including fasciclin-like arabinogalactan protein genes (−2.0-fold) and pectin methylesterase (−1.87-fold). Combined with the up-regulation of UDPGP described above, such differences in expression patterns under e[CO_2] suggest that clone 216 directs more of its fixed carbon to storage and/or defense in the form of cell wall compounds derived from the phenylpropanoid pathway.

Previous studies have reported that clone 216 clearly differs in leaf and wood structure compared with clone 271 (Oksanen *et al.*, 2001, 2003; Kaakinen *et al.*, 2004; Table 1). Clone 216 has significantly smaller fiber and vessel lumen diameters compared with clone 271, and clone 216 has the largest wall percentage, as well as the smallest vessel percentage, of any of the clones at the Aspen FACE site (Kaakinen *et al.*, 2004). Interestingly, there was a significant (6.91-fold) up-regulation of a NAC [for NAM (no apical meristem), Arabidopsis NAC domain containing protein (ATAF1/2) and CUC2 (cup-shaped cotyledons 2)] domain transcription factor in the leaves of

late-season clone 216 in response to e[CO_2] (Table 4). NAC domain transcription factors have been implicated as transcriptional switches that regulate secondary wall synthesis in fibers (Zhong *et al.*, 2006). Ectopic over-expression of such NAC transcription factors in the leaves of *Arabidopsis* results in the activation of the expression of secondary wall biosynthetic genes, leading to massive deposition of secondary walls in cells that are normally nonsclerenchymatous (Zhong *et al.*, 2006). As secondary walls are important carbon sinks, the up-regulation of this NAC transcription factor may be a factor controlling carbon deposition into secondary cell wall thickening in the leaves of clone 216 compared with those of clone 271.

Protein stability and nitrogen metabolism Another intriguing difference between clones 216 and 271 was the up-regulation of KTI3 in clone 271 and an opposing down-regulation of KTI3 in clone 216 in response to e[CO_2] at the early time point (Table 3; Fig. 5). This trend reverses for clone 271 later in the season (Table 5; Fig. 5). Although KTI3 is typically associated with defense responses, it participates in such functions by impacting on the fate of specific proteins (Major & Constabel, 2008). In conjunction with alterations in KTI levels, we also observed alterations in the expression levels of various proteases, as well as genes involved in the ubiquitin pathways, in both clones. In addition, there was a substantial increase in the activity of an amino acid carrier gene (12.29-fold), as well as an increase in nitrate transporter (2.11-fold) in the leaves of early-season clone 271 (Table 3). This nitrate transporter showed an even stronger response (3.69-fold increase) in late-season clone 271 and only marginal changes in clone 216 (Table S3). Together, these results suggest that e[CO_2] treatment has different impacts on protein stability and nitrogen metabolism, especially for clone 271. To test this possibility, we again turned to biochemical assays to assess the level of nitrogen resources.

The free leaf amino acid pool is a good indicator of nitrogen status as it represents the currently available pool of reduced nitrogen for growth and is representative of whole-plant nitrogen status. The response of free amino acids to growth at e[CO_2] was unclear. There was a significant interaction between clone and date ($P = 0.005$), driven by the higher amino acid levels in clone 216 in August (Fig. 6). There was also a significant interaction between CO_2 and clone ($P = 0.047$) and a marginally significant interaction between CO_2, clone and date ($P = 0.072$). Clone 216 showed no response in June, but an increase in August, whereas clone 271 showed a consistently lower amino acid content at e[CO_2] (Fig. 6). There was no effect of CO_2 or clone on protein content, but there was a significant clone × date interaction ($P = 0.009$), driven by the higher protein content in clone 216 in August. This trend was also seen in nitrate content, where there was a significantly higher content in clone 216 compared with clone 271 ($P = 0.018$), which was markedly higher in August (clone × date, $P = 0.049$). As the sucrose content is indicative of the available pool of carbon for growth, the sucrose to free amino acid ratio can provide an indication of potential shifts in carbon to nitrogen status. There was a significant effect of clone ($P = 0.039$) and a significant interaction between clone and date ($P = 0.023$), indicating a shift in the availability of reduced carbon and nitrogen metabolites. This shift was confined to clone 271 in August, and was driven by an increased availability of fixed carbon, which was not matched by a similar increase in reduced nitrogen. This contrasts with the response of clone 216, which was able to match increases in carbon availability occurring later in the season with increases in free amino acid content.

Regulation of transcription One last gene functional category that showed significant responses to e[CO_2], whilst also differing between the clones, was transcription factors. We have already pointed out the importance of DREB and NAC domain transcription factors in the regulation of stress-related genes in clone 271 and possible leaf cell wall thickness in clone 216. However, transcription factors, including those involved in systemic development, appear to play an important role in clone 271 in response to e[CO_2], whereas no changes in such factors are observed in clone 216 at either early or late season (Tables 2–5). Some of these clone 271-specific e[CO_2]-responsive transcription factors have known functions in stress responses and biomass development, including ethylene response factors (McGrath *et al.*, 2005), Myb family transcription factors involved in the development of cell size in poplar leaves and stems (Karpinska *et al.*, 2004; McGrath *et al.*, 2005) and one MADS-box gene known to participate in cell expansion in the vascular tissues of *P. tremuloides* (Cseke *et al.*, 2007). Such alterations in regulatory factors correlate well with the known growth responses in clone 271, and their lack of response in clone 216 may play a role in the e[CO_2] unresponsiveness of this clone.

Conclusions

Trees allocate assimilated carbon between growth, respiration, storage and chemical defense, and changes in resource availability, such as light, nitrogen or atmospheric [CO_2], can affect tree species quite differently. Growth-dominated species tend to allocate 'extra' carbon (relative to nitrogen) to growth, whereas differentiation-dominated species tend to allocate it to the production of carbon-based secondary compounds, including phenylpropanoid biosynthesis, and subsequent morphological differentiation, such as cell wall thickening (King *et al.*, 2005). This study has demonstrated that poplar clones 216 and 271 show distinctly different e[CO_2]-responsive genes during long-term exposure to e[CO_2]. In most cases, expression differences caused by developmental/seasonal and/or genotypic variations were greater than those triggered by e[CO_2] treatment, influencing multiple pathways that work synergistically to control the overall response to e[CO_2].

Much of this variation may be a result of the activity (or inactivity) of specific transcriptional factors that may be orchestrating overall development. Clone 271 clearly has a responsive, growth-dominated strategy that seems to be based on a 'less expensive' defense strategy, allowing it to be better adapted to respond to stress. Clone 216 appears to devote more energy towards carbon-based secondary compounds and possibly relocation of carbon to processes other than growth. Thus, we label clone 216 as having a preparative, differentiation-dominated strategy. Such strategies have been described previously to help explain the variable e[CO_2]-induced growth enhancements of different tree species. However, to our knowledge, this study is the first to provide evidence that such differences in fundamental biological strategies also occur within genotypes of the same tree species. Consequently, selection for improved productivity or carbon sequestration needs to take into account the genetically distinct responses, not only of different tree species, but also of different genotypes within a species. Selection of e[CO_2]-responsive clones, such as clone 271, may provide the best means to improve carbon sequestration potential in future plantations.

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References

Agarwal P, Agarwal P, Reddy M, Sopory S. 2006. Role of DREB transcription factors in abiotic and biotic stress tolerance in plants. *Plant Cell Reports* **25**: 1263–1274.

Ainsworth EA, Long SP. 2005. What have we learned from 15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy. *New Phytologist* **165**: 351–371.

Ainsworth EA, Rogers A. 2007. The response of photosynthesis and stomatal conductance to rising [CO_2]: mechanisms and environmental interactions. *Plant, Cell & Environment* **30**: 258–270.

Ainsworth EA, Rogers A, Leakey ADB, Heady LE, Gibon Y, Stitt M, Schurr U. 2007. Does elevated atmospheric [CO_2] alter diurnal C uptake and the balance of C and N metabolites in growing and fully expanded soybean leaves? *Journal of Experimental Botany* **58**: 579–591.

Allison DB, Page G, Beasley TM, Edwards JW. 2006. *DNA microarrays and related genomics techniques*. Boca Raton, FL, USA: Chapman and Hall/CRC Taylor and Francis Group.

Brosché M, Vinocur B, Alatalo ER, Lamminmaki A, Teichmann T, Ottow EA, Djilianov D, Afif D, Bogeat-Triboulot MB, Altman A *et al.* 2005. Gene expression and metabolite profiling of *Populus euphratica* growing in the Negev desert. *Genome Biology* **6**: R101.

Brown S. 2002. Measuring carbon in forests: current status and future challenges. *Environmental Pollution* **116**: 363–72.

Calfapietra C, Wiberly AE, Falbel TG, Linskey AR, Mugnozza GS, Karnosky DF, Loreto F, Sharkey TD. 2007. Isoprene synthase expression and protein levels are reduced under elevated O_3 but not under elevated CO_2 (FACE) in field-grown trees. *Plant, Cell & Environment* **30**: 654–661.

Churchill GA. 2002. Fundamentals of experimental design for cDNA microarrays. *Nature Genetics Supplement* **32**: 490–495.

Cox SE. 2005. Elevational gradient of neoformed shoot growth in *Populus tremuloides*. *Canadian Journal of Botany-Revue Canadienne de Botanique* **83**: 1340–1344.

Cross JM, von Korff M, Altmann T, Bartzetko L, Sulpice R, Gibon Y, Palacios N, Stitt M. 2006. Variation of enzyme activities and metabolite levels in 24 *Arabidopsis* accessions growing in carbon-limited conditions. *Plant Physiology* **142**: 1574–1588.

Cseke LJ, Ravinder N, Pandey AK, Podila GK. 2007. Identification of PTM5 protein interaction partners, a MADS-box gene involved in aspen tree vegetative development. *Gene* **391**: 209–222.

Deckmyn G, Muys B, Quijano JG, Ceulemans R. 2004. Carbon sequestration following afforestation of agricultural soils: comparing oak/beech forest to short-rotation poplar coppice combining a process and a carbon accounting model. *Global Change Biology* **10**: 1482–1491.

Déjardin A, Leple JC, Lesage-Descauses MC, Costa G, Pilate G. 2004. Expressed sequence tags from poplar wood tissues – a comparative analysis from multiple libraries. *Plant Biology* **6**: 55–64.

Dickson RE, Coleman MD, Pechter P, Karnosky DF. 2001. Growth and crown architecture of two aspen genotypes exposed to interacting ozone and carbon dioxide. *Environmental Pollution* **115**: 319–334.

Druart N, Rodríguez-Buey M, Barron-Gafford G, Sjödin A, Bhalerao R, Hurry V. 2006. Molecular targets of elevated [CO_2] in leaves and stems of *Populus deltoides*: implications for future tree growth and carbon sequestration. *Functional Plant Biology* **33**: 121–131.

Eamus D, Ceulemans R. 2001. Effects of greenhouse gases on the gas exchange of forest trees. In: Karnosky DF, Ceulemans R, Scarascia-Mugnozza GE, Innes JL, eds. *The impact of CO_2 and other greenhouse gases on forest ecosystems*. Wallingford, Oxfordshire, UK: CABI Publishing, 17–56.

Fluch S, Olmo CC, Tauber S, Stierschneider M, Kopecky D, Reichenauer TG, Matusíková I. 2008. Transcriptomic changes in wind-exposed poplar leaves are dependent on developmental stage. *Planta* **228**: 757–764.

Gupta P, Duplessis S, White H, Karnosky DF, Martin F, Podila GK. 2005. Gene expression patterns of trembling aspen trees following long-term exposure to interacting elevated CO_2 and tropospheric O_3. *New Phytologist* **167**: 129–41.

Hyvönen R, Agren GI, Linder S, Persson T, Cotrufo MF, Ekblad A, Freeman M, Grelle A, Janssens IA, Jarvis PG *et al.* 2007. The likely impact of elevated [CO_2], nitrogen deposition, increased temperature and management on carbon sequestration in temperate and boreal forest ecosystems: a literature review. *New Phytologist* **173**: 463–480.

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

Kaakinen S, Kostiainen K, Ek F, Saranpää P, Kubiske ME, Sober J, Karnosky DF, Vapaavuori E. 2004. Stem wood properties of *Populus tremuloides*, *Betula papyrifera* and *Acer saccharum* saplings after three years of treatments to elevated carbon dioxide and ozone. *Global Change Biology* **10**: 1513–1525.

Karnosky DF, Percy KE, Xiang B, Callan B, Noormets A, Mankovska B, Hopkin A, Sober J, Jones W, Dickson RE *et al.* 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, Pregitzer KS. 2006. Impacts of elevated atmospheric [CO_2] and [O_3] on northern temperate forest ecosystems: results from the Aspen FACE experiment. In: Nosberger J, Long SP, Norby RJ, Stitt M, Hendrey GR, Blum H, eds. *Managed ecosystems and CO_2 case studies, processes, and perspectives*. Berlin Heidelberg, Germany: Springer-Verlag, 213–229.

Karnosky DF, Pregitzer KS, Zak DR, Kubiske ME, Hendrey GR, Weinstein D, Nosal M, Percy KE. 2005. Scaling ozone responses of forest trees to the ecosystem level in a changing climate. *Plant, Cell & Environment* **28**: 965–981.

Karnosky DF, Zak DR, Pregitzer KS, Awmack CS, Bockheim JG, Dickson RE, Hendrey GR, Host GE, King JS, Kopper BJ *et al.* 2003. Tropospheric O_3 moderates responses of temperate hardwood forests to elevated CO_2: a synthesis of molecular to ecosystem results from the Aspen FACE project. *Functional Ecology* **17**: 289–304.

Karpinska B, Karlsson M, Srivastava M, Stenberg A, Schrader J, Sterky F, Bhalerao R, Wingsle G. 2004. MYB transcription factors are differentially expressed and regulated during secondary vascular tissue development in hybrid aspen. *Plant Molecular Biology* **56**: 255–270.

King JS, Pregitzer KS, Zak DR, Holmes WE, Schmidt K. 2005. Fine root chemistry and decomposition in model communities of north-temperate tree species show little response to elevated atmospheric CO_2 and varying soil resource availability. *Oecologia* **146**: 318–328.

King JS, Pregitzer KS, Zak DR, Karnosky DF, Isebrands JG, Dickson RE, Hendrey GR, Sober J. 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.

Kopper BJ, Lindroth RL. 2003. Effects of elevated carbon dioxide and ozone on the phytochemistry of aspen and performance of an herbivore. *Oecologia* **134**: 95–103.

Kubiske ME, Quinn VS, Marquardt PE, Karnosky DF. 2007. Effects of elevated atmospheric CO_2 and/or O_3 on intra- and interspecific competitive ability of aspen. *Plant Biology* **9**: 342–355.

Li P, Bohnert HJ, Grene R. 2007. All about FACE – plants in a high-[CO_2] world. *Trends in Plant Science* **12**: 87–89.

Liberloo M, Calfapietra C, Lukac M, Godbold D, Luos ZB, Polle A, Hoosbeek MR, Kull O, Marek M, Raines C *et al.* 2006. Woody biomass production during the second rotation of a bio-energy *Populus* plantation increases in a future high CO_2 world. *Global Change Biology* **12**: 1094–1106.

Liberloo M, Tulva I, Raïm O, Kull O, Ceulemans R. 2007. Photosynthetic stimulation under long-term CO_2 enrichment and fertilization is sustained across a closed *Populus* canopy profile (EUROFACE). *New Phytologist* **173**: 537–549.

Long SP, Ainsworth EA, Rogers A, Ort DR. 2004. Rising atmospheric carbon dioxide: plants FACE the future. *Annual Reviews of Plant Biology* **55**: 591–628.

Lukac M, Calfapietra C, Godbold DL. 2003. Production, turnover and mycorrhizal colonisation of root systems of three *Populus* species grown under elevated CO_2 (POPFACE). *Global Change Biology* **9**: 838–848.

Luo Z-B, Calfapietra C, Liberloo M, Scarascia-Mungozza G, Polle A. 2006. Carbon partitioning to mobile and structural fractions in poplar wood under elevated CO_2 (EUROFACE) and N fertilization. *Global Change Biology* **12**: 272–283.

Luo Z-B, Calfapietra C, Scarascia-Mugnozza G, Liberloo M, Polle A. 2008. Carbon-based secondary metabolites and internal nitrogen pools in *Populus nigra* under Free Air CO_2 Enrichment (FACE) and nitrogen fertilization. *Plant and Soil* **304**: 45–57.

Major IT, Constabel CP. 2008. Functional analysis of the kunitz trypsin inhibitor family in poplar reveals biochemical diversity and multiplicity in defense against herbivores. *Plant Physiology* **146**: 888–903.

Mankovska B, Percy K, Karnosky DF. 2005. Impacts of greenhouse gases on epicuticular waxes of *Populus tremuloides* Michx.: results from an open-air exposure and a natural O_3 gradient. *Environmental Pollution* **137**: 580–586.

McGrath KC, Dombrecht B, Manners JM, Schenk PM, Edgar CI, Maclean DJ, Scheible W-R, Udvardi MK, Kazan K. 2005. Repressor- and activator-type ethylene response factors functioning in jasmonate signaling and disease resistance identified via a genome-wide screen of *Arabidopsis* transcription factor gene expression. *Plant Physiology* **139**: 949–959.

Meehl GA, Stocker TF, Collins WD, Friedlingstein P, Gaye AT, Gregory JM, Kitoh A, Knutti R, Murphy JM, Noda A *et al.* 2007. Global climate projections. In: Solomon S, Qin D, Manning M, Chen Z, Marquis M, Averyt KB, Tignor M, Miller HL, eds. *Climate change 2007: the physical science basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge, UK and New York, NY, USA: Cambridge University Press, 747–845.

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

Noormets A, Sober A, Pell EJ, Dickson RE, Podila GK, Sober J, Isebrands JG, Karnosky DF. 2001a. Stomatal and nonstomatal 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, DeLucia EH, Gielen B, Calfapietra C, Giardina CP, King JS, Ledford J, McCarthy HR, Moore DJP, Ceulemans R *et al.* 2005. Forest response to elevated CO_2 is conserved across a broad range of productivity. *Proceedings of the National Academy of Sciences, USA* **102**: 18 052–18 056.

Nowak RS, Ellsworth DS, Smith SD. 2004. Functional responses of plants to elevated atmospheric CO_2 – do photosynthetic and productivity data from FACE experiments support early predictions? *New Phytologist* **162**: 253–280.

Oksanen E, Häikiö E, Sober J, Karnosky DF. 2003. Ozone-induced H_2O_2 accumulation in field-grown aspen and birch is linked to foliar ultrastructure and peroxisomal activity. *New Phytologist* **161**: 791–799.

Oksanen E, Sober J, Karnosky DF. 2001. Impacts of elevated CO_2 and/or O_3 on leaf ultrastructure of aspen (*Populus tremuloides*) and birch (*Betula papyrifera*) in the Aspen FACE experiment. *Environmental Pollution* **115**: 437–446.

Osier TL, Lindroth RL. 2006. Genotype and environment determine allocation to and costs of resistance in quaking aspen. *Oecologia* **148**: 293–303.

Perlack RD, Wright LL, Turhollow AF, Graham RL, Stokes BJ, Erbach DC. 2005. Biomass as feedstocks for a bioenergy and bioproducts industry: the technical feasibility of a billion-ton annual supply. ORNL/TM-2005/66.

Prentice IC. 2001. The carbon cycle and atmospheric carbon dioxide. In: Houghton JT, Ding Y, Griggs DJ, Noguer M, van der Linden PJ, Dai X, Maskell K, Johnson CA, eds. *Climate change 2001: the scientific basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge, UK and New York, NY, USA: Cambridge University Press, 183–238.

Riikonen J, Kets K, Darbah J, Oksanen E, Sober A, Vapaavuori E, Kubiske ME, Nelson N, Karnosky DF. 2008. Carbon gain and bud physiology in *Populus tremuloides* and *Betula papyrifera* grown under long-term exposure to elevated concentrations of CO_2 and O_3. *Tree Physiology* **28**: 243–254.

Rinaldi C, Kohler A, Frey P, Duchaussoy F, Ningre N, Couloux A, Wincker P, Le Thiec D, Fluch S, Martin F *et al.* 2007. Transcript profiling of poplar leaves upon infection with compatible and incompatible strains of the foliar rust *Melampsora larici-populina*. *Plant Physiology* **144**: 347–366.

Rogers A, Gibon Y, Stitt M, Morgan PB, Bernacchi CJ, Ort DR, Long SP. 2006. Increased carbon availability at elevated carbon dioxide concentration improves nitrogen assimilation in a legume. *Plant, Cell & Environment* **29**: 1651–1658.

Sims REH, Hastings A, Schlamadinger B, Taylor G, Smith P. 2006. Energy crops: current status and future prospects. *Global Change Biology* **12**: 2054–2076.

Taylor G, Street NR, Tricker PJ, Sjodin A, Graham L, Skogstrom O, Calfapietra C, Scarascia-Mugnozza G, Jansson S. 2005. The transcriptome of *Populus* in elevated CO_2. *New Phytologist* **167**: 143–154.

Taylor G, Tallis MJ, Giardina CP, Percy KE, Miglietta F, Gupta PS, Gioli B, Calfapietra C, Gielen B, Kubiske ME *et al.* 2008. Future atmospheric CO_2 leads to delayed autumnal senescence. *Global Change Biology* **14**: 264–275.

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

Tsai C-J, Harding SA, Tschaplinski TJ, Lindroth RL, Yuan Y. 2006. Genome-wide analysis of the structural genes regulating defense phenylpropanoid metabolism in *Populus*. *New Phytologist* **172**: 47–62.

Tuskan GA, Difazio S, Jansson S, Bohlmann J, Grigoriev I, Hellsten U, Putnam N, Ralph S, Rombauts S, Salamov A *et al.* 2006. The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). *Science* **313**: 1596–604.

Wustman BA, Oksanen E, Karnosky DF, Sober 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.

Zhong R, Demura T, Ye ZH. 2006. Links SND1, a NAC domain transcription factor, is a key regulator of secondary wall synthesis in fibers of Arabidopsis. *Plant Cell* **18**: 3158–70.

Zimmermann P, Schildknecht B, Craigon D, Garcia-Hernandez M, Gruissem W, May S, Mukherjee G, Parkinson H, Rhee S, Wagner U *et al.* 2006. MIAME/Plant – adding value to plant microarray experiments. *Plant Methods* **2**: 1 doi:10.1186/1746-4811-2-1

Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Scatter plots comparing the uniformity of data passing a coefficient of variance (CV) < 0.25 from normalized transcript intensities for ~25 000 cDNA probes prepared from clones 216 and 271 RNA extracts from both early (June) and late (August) time points.

Table S1 List of primers used in quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) analysis.

Table S2 Expanded dataset for clone 216 June expression information (including comparisons between clones and experiments).

Table S3 Expanded dataset for clone 271 June expression information (including comparisons between clones and experiments).

Table S4 Expanded dataset for clone 216 August expression information (including comparisons between clones and experiments).

Table S5 Expanded dataset for clone 271 August expression information (including comparisons between clones and experiments).

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