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Increasing atmospheric CO_2 reduces metabolic and physiological differences between isoprene- and non-isoprene-emitting poplars

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Summary

- Isoprene, a volatile organic compound produced by some plant species, enhances abiotic stress tolerance under current atmospheric CO_2 concentrations, but its biosynthesis is negatively correlated with CO_2 concentrations. We hypothesized that losing the capacity to produce isoprene would require stronger up-regulation of other stress tolerance mechanisms at low CO_2 than at higher CO_2 concentrations.
- We compared metabolite profiles and physiological performance in poplars (*Populus* × *canescens*) with either wild-type or RNAi-suppressed isoprene emission capacity grown at pre-industrial low, current atmospheric, and future high CO_2 concentrations (190, 390 and 590 ppm CO_2, respectively).
- Suppression of isoprene biosynthesis led to significant rearrangement of the leaf metabolome, increasing stress tolerance responses such as xanthophyll cycle pigment de-epoxidation and antioxidant levels, as well as altering lipid, carbon and nitrogen metabolism. Metabolic and physiological differences between isoprene-emitting and suppressed lines diminished as growth CO_2 concentrations rose.
- The CO_2 dependence of our results indicates that the effects of isoprene biosynthesis are strongest at pre-industrial CO_2 concentrations. Rising CO_2 may reduce the beneficial effects of biogenic isoprene emission, with implications for species competition. This has potential consequences for future climate warming, as isoprene emitted from vegetation has strong effects on global atmospheric chemistry.

Introduction

Atmospheric CO_2 concentrations have varied considerably in geological time (Beerling & Royer, 2011). CO_2 concentrations have increased from 280 ppm in the middle of the 19th Century to current values of *c.* 395 ppm (Leuenberger *et al.*, 1992; Tans & Keeling, 2012). Anthropogenic fossil fuel use and land use change are expected to continue increasing atmospheric CO_2 concentrations, with concentrations of up to 1020 ppm predicted for the year 2100 (Meehl *et al.*, 2007). Low atmospheric CO_2 concentrations during and succeeding the Last Glacial Maximum are thought to have imposed selective pressures on organisms, and acted as a key driver in the evolution of plant traits such as C_4 photosynthesis (Ehleringer *et al.*, 1991; Osborne & Sack, 2012; Sage *et al.*, 2012). The higher atmospheric CO_2 concentrations of the current time and those predicted for the future may negate the advantage of such traits, including that of C_4 plants over their C_3 competitors (Sage & Kubien, 2003).

While photosynthesis is directly affected by CO_2 concentrations through substrate availability for the Calvin–Benson cycle, CO_2 concentrations also impact other leaf physiological processes, such as isoprene emission rates (Wilkinson *et al.*, 2009; Possell & Hewitt, 2011). Isoprene (2-methyl-1,3-butadiene) is a volatile organic chemical that is emitted by some, but not all, plant species and is synthesized in chloroplasts through the 2-C-methyl D-erythritol 4-phosphate (MEP) pathway (Harley *et al.*, 1999; Monson *et al.*, 2013). Isoprene biosynthesis is negatively correlated with atmospheric CO_2 concentration (Rosenstiel *et al.*,

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2003; Wilkinson *et al.*, 2009; Possell & Hewitt, 2011), and the multiple independent gains and losses of isoprene emission in the plant kingdom may be linked, in part, to swings in the geological history of atmospheric CO_2 concentrations (Monson *et al.*, 2013). Isoprene is one of the most abundant hydrocarbons emitted by plants, and these emissions play an important role in modifying atmospheric chemistry (Fuentes *et al.*, 2000), particularly in extending the lifespan of methane (Poisson *et al.*, 2000; Archibald *et al.*, 2011) and in contributing to the formation of secondary organic aerosols (Kiendler-Scharr *et al.*, 2012).

Although the exact biochemical or biophysical mechanism(s) is unknown, isoprene increases photosynthetic tolerance to the oxidative stresses most frequently produced during periods of high leaf heat loads, high photosynthetic photon flux densities, low soil water availability and low atmospheric CO_2 concentrations (Sharkey & Singsaas, 1995; Behnke *et al.*, 2007, 2010a; Vickers *et al.*, 2009; Way *et al.*, 2011). Isoprene increases the stability of photosynthetic processes associated with chloroplast thylakoids (Velikova *et al.*, 2011) and reacts with reactive oxygen species (ROS; Jardine *et al.*, 2012), with both mechanisms potentially providing increased tolerance of abiotic stresses. Photosynthesis is more susceptible to stress caused by high temperature and high photon flux densities at low atmospheric CO_2 concentrations because photosynthetic carbon reduction rates are reduced: the reduced sink capacity of the Calvin–Benson cycle at low CO_2 increases the need for alternative mechanisms, such as isoprene production, that can either dissipate excess excitation energy through nonphotochemical processes or remove oxidative radical species that result from excess energy in the photosynthetic apparatus (Behnke *et al.*, 2010a). Because isoprene's effect is dose-dependent (Singsaas *et al.*, 1997), isoprene-based photosynthetic stress tolerance is also greater at low CO_2 than at high CO_2 (Way *et al.*, 2011). Any advantage in maintaining photosynthetic carbon gain at low CO_2 may be offset by the cost of isoprene biosynthesis, which amounts to 10 moles of CO_2, 24 moles of ATP and 14 moles of NADPH for each mole of isoprene produced (Niinemets *et al.*, 1999). However, isoprene-emitting leaves grown at low CO_2 had both higher net photosynthetic rates and lower dark respiration rates than non-emitting leaves, differences that more than compensated for the carbon cost of isoprene (Way *et al.*, 2011).

If higher growth CO_2 concentrations minimize phenotypic and metabolic differences between emitting and non-emitting plants, there could be reduced selection pressure on the trait of isoprene biosynthesis in a high-CO_2 world. Because biogenic isoprene emissions impact climate (Poisson *et al.*, 2000; Archibald *et al.*, 2011; Kiendler-Scharr *et al.*, 2012), shifts in the abundance of isoprene-emitting species or their emission rates across geologically relevant atmospheric CO_2 concentrations could have significant effects on global atmospheric chemistry (Pacifico *et al.*, 2012). With this context in mind, we therefore examined how low (190 ppmv), current ambient (390 ppmv), and elevated (590 ppmv) growth CO_2 concentrations affected leaf metabolism and photosynthetic physiology in isoprene-emitting (wild-type (WT) and empty vector control (C)) poplar (*Populus* × *canescens*) lines and lines with suppressed isoprene biosynthesis (RA2 and RA22). We hypothesized that: (1) suppression of isoprene biosynthesis would cause the largest metabolic and physiological changes at pre-industrial CO_2 concentrations, with smaller differences between emitting and non-emitting lines at current and future predicted CO_2 concentrations; and (2) metabolic changes in suppressed lines compared with emitting lines would involve increases in abiotic stress tolerance mechanisms to compensate for the loss of isoprene-related stress tolerance.

Materials and Methods

Plant material

We used four lines of *Populus* × *canescens* (Aiton) Sm. (syn. *Populus tremula* × *P. alba*): two isoprene-emitting lines (WT and the control for the transgenic manipulation (C)) and two non-isoprene-emitting transgenic lines where isoprene synthase expression was silenced by RNA interference (RNAi); for more details of the plant lines, see Behnke *et al.* (2007). We chose two lines (RA2 and RA22), out of 10 transgenic lines, where isoprene emission rates were most suppressed (see Behnke *et al.*, 2007). Additionally, three independent transgenic *PcISPS*:*GFP* lines of *Populus* × *canescens* (nine trees), in which the *PcISPS* (*P. canescens* isoprene synthase) promoter is fused to the enhanced green fluorescent protein (GFP) reporter gene (see details in Cinege *et al.*, 2009), were used for imaging of *ISPS* promoter activity at different CO_2 concentrations.

Detailed growth conditions of the experiment can be found in Way *et al.* (2011). Briefly, cuttings were established on misting benches in the Duke University Phytotron. Once roots had formed, they were planted in 1 : 1: 1 (v/v/v) sand : perlite : peat in 10 × 10 × 36 cm pots, and five or more plants per line (WT, C, RA2 and RA22) were moved into each of three growth chambers (Model M-13; Environmental Growth Chambers, Chagrin Falls, OH, USA). Chambers were set for low (190 ppm), ambient (390 ppm) or high (590 ppm) CO_2 concentration, as measured with an infrared gas analyzer (LI-COR 6252; Li-Cor, Lincoln, NE, USA) every 2–5 min. Elevated CO_2 was attained by injecting pure CO_2 into the ambient airstream as needed, and low CO_2 was achieved by scrubbing CO_2 from the incoming air with soda lime. Treatments were rotated between chambers every 3 wk to minimize chamber effects. All chambers supplied 700 μmol photons m^{-2} s^{-1} at canopy height over a 16-h photoperiod, and 27 : 23°C day : night temperatures. Plants were fertilized weekly with half-strength Hoagland's solution. Measurements were made on plants that had been exposed to treatments for 10–13 wk; there were no obvious size differences between plants from the different lines.

Gas exchange

Gas exchange and isoprene emission rates were assessed at growth CO_2 concentrations (190, 390 and 590 ppm) at 30°C leaf temperature, saturating light (1000 μmol photons m^{-2} s^{-1}) and 50% relative humidity using a portable photosynthesis system (Li-6400; Li-Cor). A fraction of the outgoing cuvette air was

diverted to a chemiluminescence-based fast isoprene sensor (Hills Scientific, Boulder, CO, USA; see Hills *et al.*, 1991). Measurements were made on the ninth leaf from the top of five trees from each of the WT, C and suppressed lines.

Confocal scanning laser microscopy

Leaf cross-sections were sampled from the ninth or tenth leaf from the top of plants carrying GFP fused to the *PcISPS* promoter (Cinege *et al.*, 2009). Three independent GFP lines from each growth CO_2 concentration were analyzed, with three sections taken from each of the nine trees. Sections were examined using a confocal laser-scanning microscope (Zeiss LSM 510 upright confocal and LSM Image Browser software; Zeiss, Jena, Germany). Setting details for images are as described in Cinege *et al.* (2009). Images were divided into five cell layers (upper and lower palisade cell layers and upper, middle and lower spongy mesophyll layers) by manually tracing cell outlines on-screen (ImageJ; US National Institutes of Health, Bethesda, MD, USA). Chlorophyll autofluorescence and GFP fluorescence intensity were analyzed in each cell layer for each image (ImageJ).

Analysis of photosynthetic pigments

The ninth leaf from the tree apex was harvested from five plants per line per CO_2 concentration between 11:00 and 12:20 h, immediately frozen in liquid N_2 and kept at −80°C until analysis. Pigment extractions and analyses were performed as described in Behnke *et al.* (2007). Briefly, 50 mg of frozen leaf material was extracted for 10 min in darkness at room temperature with 1 ml of acetone, then centrifuged for 10 min at 15 000 ***g*** and 4°C. The pellet was re-extracted with 500 µl of acetone. Both supernatants were unified and pigments were measured with a HPLC system (Model 515 pump, 717 cooled autosampler; both from Waters, Milford, MA, USA) using a UV/visible diode-array detector at 440 nm wavelength (Model 2996 and 447; Waters). The same leaf samples were used in the nontargeted metabolite and fatty acid analyses.

Nontargeted metabolite analysis

Nontargeted metabolite analysis was performed using a Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-MS; APEX Qe; Bruker, Bremen, Germany) equipped with a 12-Tesla superconducting magnet and an Apollo II electrospray (ESI) source (Bruker). Metabolite identification was achieved via the MassTRIX web site (http://metabolomics.helmholtz-muenchen.de/masstrix2/; Suhre & Schmitt-Kopplin, 2008) by using KEGG/API (http://www.genome.jp/kegg/soap/). Details can be found in Behnke *et al.* (2010b) with the following modification: mass spectra were acquired with longer time domain transients (two MWords instead of one in the previous study) to achieve higher resolution, obtaining a mass resolving power of 200 000 at $m/z = 400$, sufficient for biological samples. Ions were produced only in the negative ionization mode of electrospray and the masses were corrected against H^+ loss. Peak mass intensities (threshold of 1e6) were exported to a peak list at a signal to noise (S/N) ratio of 2 and submitted for annotation to an automated database search using MassTRIX; the maximal error accepted was 3 ppm, the database chosen for annotation was KEGG with expanded lipids (HMDB, LipidMaps; v. 06-2009), and the organism selected was *Populus trichocarpa.*

Phospholipid fatty acid extraction

Phospholipid fatty acids (PLFAs) were extracted from frozen leaves, using a procedure modified after Cho *et al.* (1992). In brief, 50 mg of frozen leaf material was extracted with $CHCl_3 : CH_3OH : 1$ N HCl (40 : 80 : 1, v/v/v), after centrifugation at 5000 ***g***, and subsequently washed with water and a solution of 0.5 M HCl in $CH_3OH : H_2O$ (1 : 1, v/v). The lipid extract was further prepared for analysis after Zelles *et al.* (1995). After separating the phospholipid fraction from neutral lipids and glycolipids on a silica bonded phase column (SPE-SI 2 g 12 ml^{-1}; Bond Elut; Agilent Technologies, Palo Alto, CA, USA), fatty acid methyl esters (FAMEs) were obtained after mild alkaline hydrolysis and prepared for GC separation using myristic FAME as an internal standard. FAMEs were measured using a 5973MSD GC-MS (Agilent Technologies) linked via a combustion unit to an isotope ratio mass spectrometer (DeltaPlus; Thermo Electron Cooperation, Bremen, Germany) and identified via established fatty acid libraries and characteristic retention times. Fatty acids are designated as the total number of C atoms followed by the number of double bonds and their location (ω) after the colon. Saturated straight-chain fatty acids are indicated by 'n'.

Statistics

Confocal microscopy data were analyzed with a two-way ANOVA using growth CO_2 treatment and cell layer (JMP Pro 9; SAS Institute, Cary, NC, USA). There were no differences between the two emitting and two non-emitting lines in any gas exchange parameter across the CO_2 treatments (two-way ANOVA and post hoc Tukey test ($P > 0.05$)), so gas exchange data were analyzed with a two-way ANOVA based on isoprene emission capability and growth/measurement CO_2 concentration (JMP Pro 9).

Metabolomic differences and similarities among samples were revealed using principal component analysis (PCA) and validated by 'full cross-validation' using the software package The Unscrambler (v. 8.0; CAMO A/S, Oslo, Norway). The peak list of FT-ICR-MS mass intensities was selected as the *X* variable (scaled with 1 SD^{-1} to have the same unit variance) and all samples (i.e. $n = 60$) were used. Differences in metabolites among the four lines (WT, C, RA2 and RA22) were discovered by cluster analysis (Hierarchical Clustering Explorer (HCE) v3.0; http://www.cs.umd.edu/hcil/hce/). Only masses showing opposite profiles between the two clustered WT/C and RA lines (resulting in 10 individual samples per CO_2 concentration for emitters and for non-emitters; average linkage cluster method using Euclidean distance measurements to assess similarity/

difference) with a 0.8 Pearson product-moment correlation coefficient threshold were taken into account.

To assess the significance of changes in metabolite profiles, a Student's *t*-test ($P < 0.05$) was performed applying a false discovery rate (FDR) of 5% according to the Benjamini Hochberg modified correction (Benjamini & Hochberg, 1995; Benjamini *et al.*, 2006; MATLAB R2011b; MathWorks, Natick, MA, USA). For each change in a metabolite concentration that was significant in at least one CO_2 treatment, the $\log_2$ ratios of peak intensities between WT/C and RA2/RA22 were calculated ($n = 10$), based on separation in the PCA analysis. The statistical significance of differences between WT/C and RA lines and CO_2 concentrations in pigments and fatty acids was determined with two-way ANOVAs and post hoc Tukey tests ($P < 0.05$; SIGMAPLOT 11.0; Systat Software Inc., San Jose, CA, USA).

Results

Isoprene synthase promoter (*PcISPS:GFP*) activity, measured as GFP fluorescence intensity, was negatively correlated with growth CO_2 concentration (Fig. 1a–f), and showed a cell-specific enhancement at low, but not higher, CO_2 concentrations (Fig. 1d–f). *PcISPS:GFP* activity was significantly correlated with isoprene emission rates in WT/C plants grown in the three different growth CO_2 concentrations ($r^2 = 0.994$; $P = 0.050$; Fig. 1g), with both the greatest promoter activity and the highest isoprene emission rates at the lowest CO_2 concentration. In accordance with previous observations (Behnke *et al.*, 2007; Way *et al.*, 2011), isoprene emission rates were negligible and independent of CO_2 concentration in RA lines, but negatively correlated with CO_2 concentration in WT/C lines (Fig. 2d).

Chlorophyll autofluorescence was also greatest at low CO_2 concentrations, peaking in the same cell layer as *PcISPS:GFP* activity (first palisade cells adjacent to the spongy parenchyma; Fig. 1). However, chlorophyll autofluorescence and net photosynthetic rates in the WT/C lines were negatively correlated ($r^2 = 0.686$; $P = 0.035$; Fig. 1h), with strong chlorophyll autofluorescence but low photosynthetic rates at the lowest CO_2 concentration. WT/C poplars had higher net CO_2 assimilation rates than RA lines at low ($P < 0.0001$) and ambient CO_2 ($P < 0.03$), but these differences were not present at the highest CO_2 concentration (Fig. 2a,b). Net photosynthetic rates increased and stomatal conductance decreased with measurement CO_2 concentration, but there was no difference among lines in the ratio of intercellular to ambient CO_2 concentrations (C_i/C_a) in plants grown in any of the three CO_2 concentrations ($P > 0.05$; Fig. 2a–c). Chlorophyll concentrations were positively correlated with chlorophyll autofluorescence across growth CO_2 concentrations, and negatively correlated with net photosynthetic rates ($r^2 = 0.548$; $P = 0.026$; Fig. 1h), as net photosynthetic rates were constrained most at low atmospheric CO_2 when CO_2 substrate concentrations limit carbon uptake. *PcISPS:GFP* activity and chlorophyll autofluorescence were positively correlated across all CO_2 concentrations and cell layers ($r^2 = 0.737$; statistical difference: chlorophyll fluorescence: $P < 0.001$; GFP fluorescence: $P = 0.067$; Supporting Information Fig. S1).

Nontargeted metabolomic and PLFA analyses

For the nontargeted metabolite profile, leaves that developed at the lowest CO_2 concentration formed a distinct group from leaves grown at ambient and elevated CO_2 concentrations (Fig. 3). Results from a PCA showed that WT/C lines grouped separately from RA lines, with a stronger separation between lines in samples from the lowest CO_2 concentration. Significant metabolic differences (*P*-values corrected to an FDR of 5%) between WT/C and RA lines were indicated for almost 1100 individual masses (Fig. S2), of which 60 (Table S1) could be identified through the KEGG database with the MassTRIX approach (Suhre & Schmitt-Kopplin, 2008). These identified metabolites were associated with numerous biochemical pathways, including carbohydrate, nitrogen, fatty acid, terpenoid, and ubiquinone metabolism (Fig. 4, Table S1). Metabolites associated with nucleotide and ubiquinone metabolism, and amino acids and other compounds related to nitrogen metabolism were enhanced in the leaves of RA plants. Sucrose and sorbitol, two key transport forms of carbohydrates, were also enhanced in RA leaves, but sugars related to the pentose phosphate cycle and glycolysis/gluconeogenesis (i.e. D-ribose, salicin 6-phosphate, phospho-D-glycerate and 6-phospho-D-gluconate) were enhanced in WT/C leaves compared with leaves from the RA lines. Calcitriol was reduced in the leaves of RA plants, especially those grown at the lowest CO_2 concentration. Despite a stronger restructuring of the metabolome in non-isoprene emitting leaves at low CO_2 than at higher CO_2 concentrations, some metabolites (such as dodecanoid and dihydroxyhexadecanoic acids) were positively correlated with CO_2 concentration (Fig. 4).

Across all pathways, concentrations of antioxidants (e.g. ascorbic acid and glutathione) were greater in the leaves of RA plants compared with WT/C plants, with larger differences between these lines at the lowest CO_2 concentration (Fig. 4). Concentrations of thiamin monophosphate were also higher in RA leaves, compared with WT/C leaves, and greatly increased when plants were grown at the lowest CO_2 concentration (Table S1). Growth at the lowest CO_2 concentration stimulated photosynthetic pigment production in all lines, and de-epoxidized xanthophyll cycle pigment concentrations in leaves of RA lines, compared with higher CO_2 concentrations (Table S2; Figs 1h, 5).

There were large differences in fatty acid metabolism between emitting and suppressed lines across the CO_2 treatments (Fig. 4). Phosphatidic acid was up-regulated in leaves grown at the lowest CO_2 concentration in RA lines (Fig. 4, Table S1). Because isoprene has been shown to reduce thylakoid trans-membrane leakage of protons (Velikova *et al.*, 2011), we analyzed the PLFA content in more detail using GC-MS techniques. Leaves of RA lines had greater concentrations of unsaturated fatty acids (such as α-linoleic acid and α-linolenic acid) in their PLFAs than WT/C lines (Fig. 6). Suppression of isoprene emission significantly increased ($P < 0.05$) the content of 16:1, 18:1 and 18:3 PLFA when plants were grown at low CO_2 compared with higher CO_2 concentrations (Table S2). Within individual fatty acid species, differences between lines

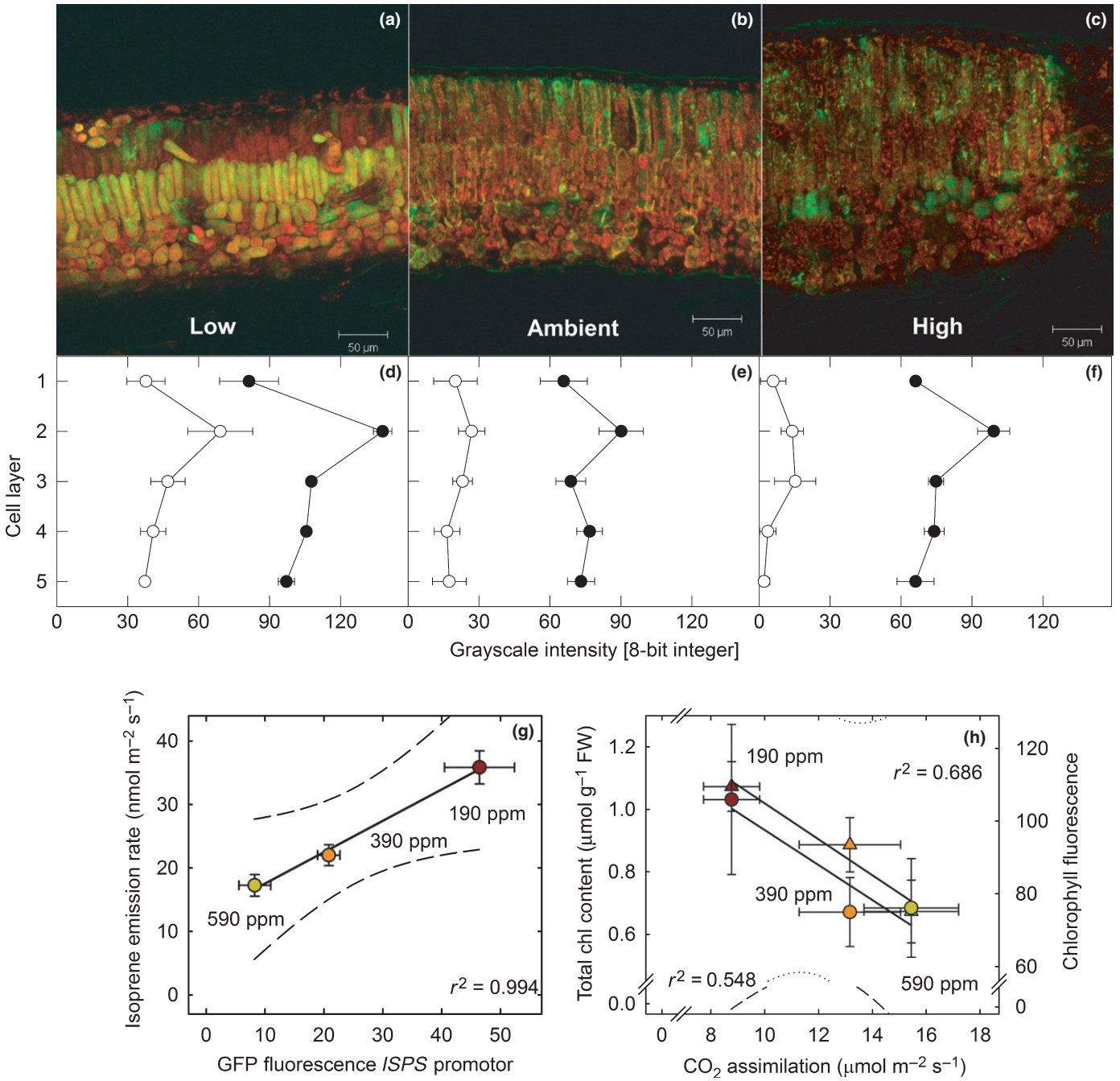

Fig. 1 (a–c) Poplar (*Populus* × *canescens*) representative leaf cross-sections from (a) low (190 ppm), (b) ambient (390 ppm), and (c) high (590 ppm) growth CO_2 concentrations, showing red chlorophyll autofluorescence and green GFP fluorescence (indicating isoprene synthase (*ISPS*) promoter activity). (d–f) Chlorophyll autofluorescence intensity (closed symbols) and ISPS activity (measured by GFP intensity; open symbols) across leaf cell layers in isoprene-emitting lines grown at (d) low (190 ppm), (e) ambient (390 ppm), and (f) high (590 ppm) CO_2 concentrations. Cell layers: 1, upper/adaxial palisade cells; 2, lower/abaxial palisade cells; 3, upper/adaxial spongy mesophyll cells; 4, middle spongy mesophyll cells; 5, lower/abaxial spongy mesophyll cells. Values are mean ± SE, $n = 3$ trees per CO_2 concentration, with three sections per tree. (g, h) Relationships between (g) mean leaf cross-sectional GFP fluorescence and leaf isoprene emission rate; (h) mean net CO_2 assimilation rate and both mean leaf cross-sectional chlorophyll autofluorescence (circles) and leaf chlorophyll concentration (triangles). Values are mean ± SE; for chlorophyll and GFP fluorescence, $n = 3$ trees, with three sections per tree; for isoprene emission and net CO_2 assimilation rate, $n = 10$ trees; for chlorophyll concentration, $n = 5$ leaves.

were most pronounced when trees were grown at the lowest CO_2 concentration (Fig. 6a). The percentage of unsaturated fatty acids and the double bond index in the PLFA were negatively correlated with growth CO_2 concentration in RA plants (Fig. 6b), but there was no CO_2-dependent trend in WT/C plants. There was also a CO_2-dependent increase of oxylipin metabolites in RA leaves, including the lipid-based hormone signals jasmonic acid and methyl jasmonate (Fig. 4).

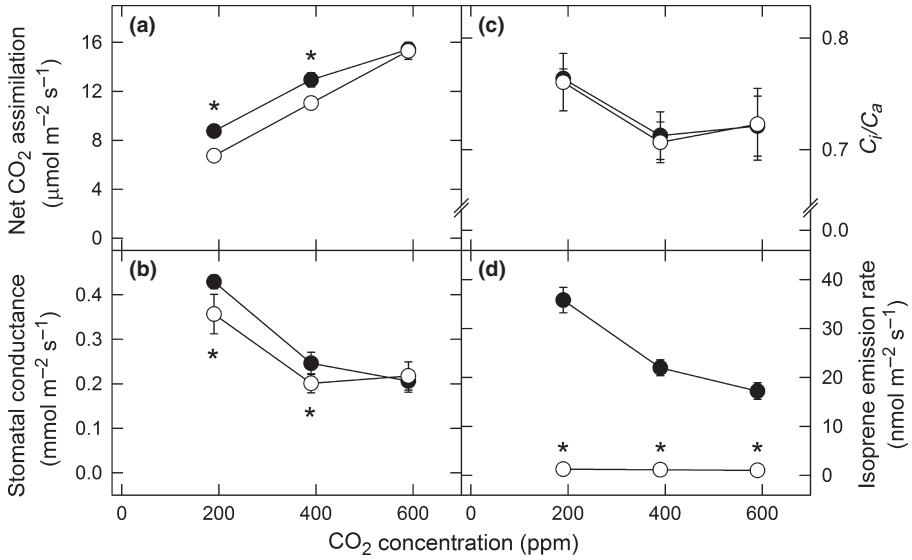

Fig. 2 Effect of growth CO_2 concentration on: (a) net CO_2 assimilation rate; (b) stomatal conductance; (c) the ratio of intercellular to ambient CO_2 concentration (C_i/C_a); and (d) isoprene emission rate in isoprene-emitting (closed symbols) and isoprene emission suppressed (open symbols) poplar lines. Measurements were made at growth CO_2 concentration, 30°C leaf temperature and 1000 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD). Values are mean ± SE, $n = 10$ trees. Asterisks indicate significant differences between lines (one-way ANOVA; $P < 0.05$).

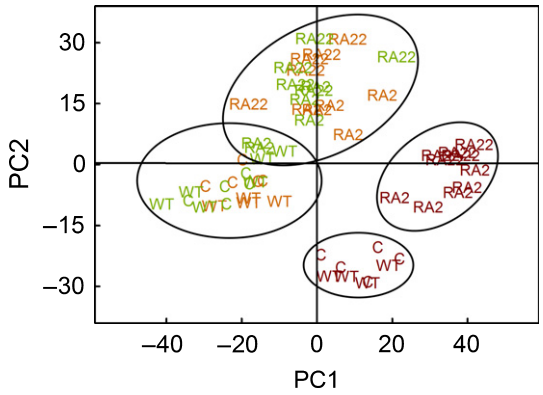

Fig. 3 Principal component analysis (PCA) of metabolomic data (*x*-axis = PC1 = 28%; *y*-axis = PC2 = 15%) from isoprene-emitting (wild-type (WT)/control (C)) and isoprene emission suppressed (RA2/RA22) poplar lines analyzed by nontarget Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-MS). Colors represent different CO_2 concentrations (red, 190 ppm; orange, 390 ppm; green, 590 ppm).

Discussion

Increasing CO_2 concentrations diminished metabolic and physiological differences between isoprene- and non-isoprene-emitting poplars, in support of our first hypothesis. The effects of isoprene suppression on leaf metabolism were stronger at low CO_2 concentration and gradually diminished as the atmospheric CO_2 concentration was increased, in agreement with the known negative correlation between isoprene biosynthesis and CO_2 concentration (Wilkinson *et al.*, 2009; Possell & Hewitt, 2011; Fig. 7a). This divergence between the metabolic phenotypes of the lines highlights the extensive remodeling of cellular and physiological processes that occurs in the absence of isoprene biosynthesis, in particular at low CO_2 concentration (Fig. 7b–d). In accordance with our second hypothesis, plants with suppressed isoprene biosynthesis grown at lower CO_2 concentrations up-regulated compensatory stress tolerance mechanisms, as well as various compounds related to isoprene function or isoprenoid biosynthesis, but these effects were less apparent at high CO_2 concentrations.

Changes in leaf metabolism in response to suppression of isoprene biosynthesis are negatively correlated with CO_2 concentration

The large metabolomic difference between RA lines and WT/C plants showed that suppression of isoprene biosynthesis has profound, CO_2-dependent consequences for many more metabolites than may be expected on the basis of the loss of isoprene function. This is probably a consequence of a reduction in C fluxes through the MEP pathway and the negative correlation of isoprene biosynthesis with CO_2 concentration. In poplar, isoprene biosynthesis is by far the main C sink of the plastidic MEP pathway (Sharkey & Yeh, 2001): C flux through the MEP pathway is *c.* 100-fold larger in isoprene-emitting than in non-emitting poplar and is negatively correlated to CO_2 concentration (A. Ghirardo *et al.*, unpublished data). The MEP pathway produces many key compounds involved in the synthesis and maintenance of the photosynthetic apparatus (e.g. carotenoids, plastoquinone and chlorophylls), antioxidant molecules (i.e. tochopherols; Munné-Bosch, 2005), phytohormones involved in plant development (giberellin) or stomatal closure (abscisic acid (ABA)), and the isoprenyl moiety for protein isoprenylation (Gerber *et al.*, 2009). Thus, a perturbation of C fluxes when *ISPS* is suppressed explains the wide metabolic effects observed. Moreover, because there is cross-talk between the MEP pathway and the cytosolic isoprenoid mevalonic (MVA) pathway (Laule *et al.*, 2003), perturbation of the MEP pathway could affect cytosolic metabolites such as phytosterols, dolichols, and farnesyl residues, with broad implications for cell membrane signal transduction, cellular sorting and cytoskeleton reorganization (Crowell, 2000).

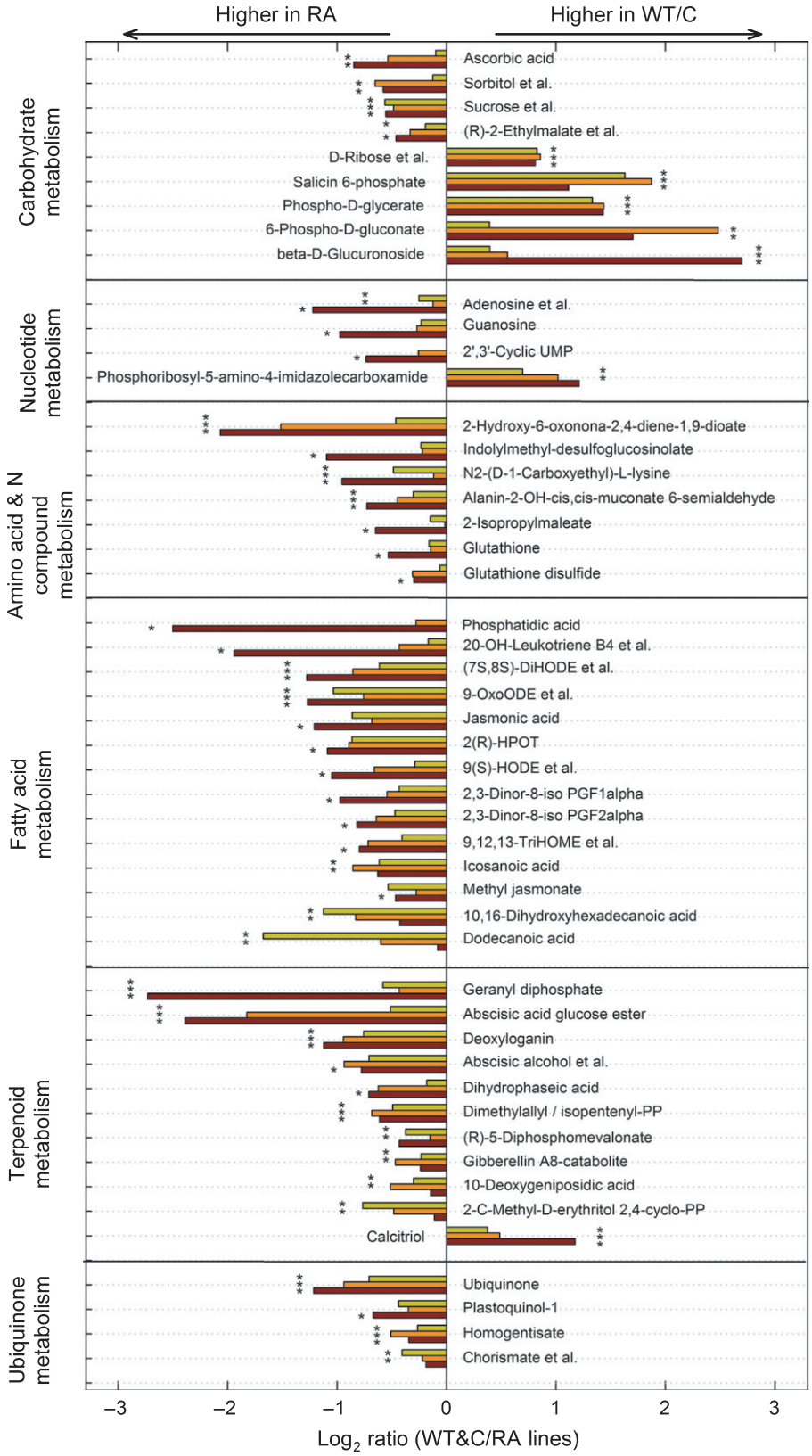

Fig. 4 Metabolomic differences between leaves from both isoprene-emitting lines (wild-type (WT)/control (C)) and both isoprene emission suppressed (RA) poplar lines grown at different CO_2 concentrations (red, 190 ppm; orange, 390 ppm; green, 590 ppm), analyzed by nontarget Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-MS). Identifications were obtained by comparing the results with annotated masses of each compound in KEGG databases and relating them to the main metabolism (for multiple assignments, only one name is reported as 'name sum' + et al.; the complete list can be found in Supporting Information Table S1). An asterisk over a bar indicates a significant difference between WT/C and RA2/RA22 within that CO_2 growth concentration (*t*-test; $P < 0.05$; mean ± SE; $n = 10$).

When *ISPS* was suppressed, decreased demand for C entering the MEP pathway resulted in a strong metabolic shift across multiple pathways, with the strongest overall effects at low CO_2, where isoprene synthesis is normally highest. A direct effect from isoprene suppression is the accumulation of the isoprene precursor dimethylallyl diphosphate (DMADP; Behnke *et al.*, 2007)

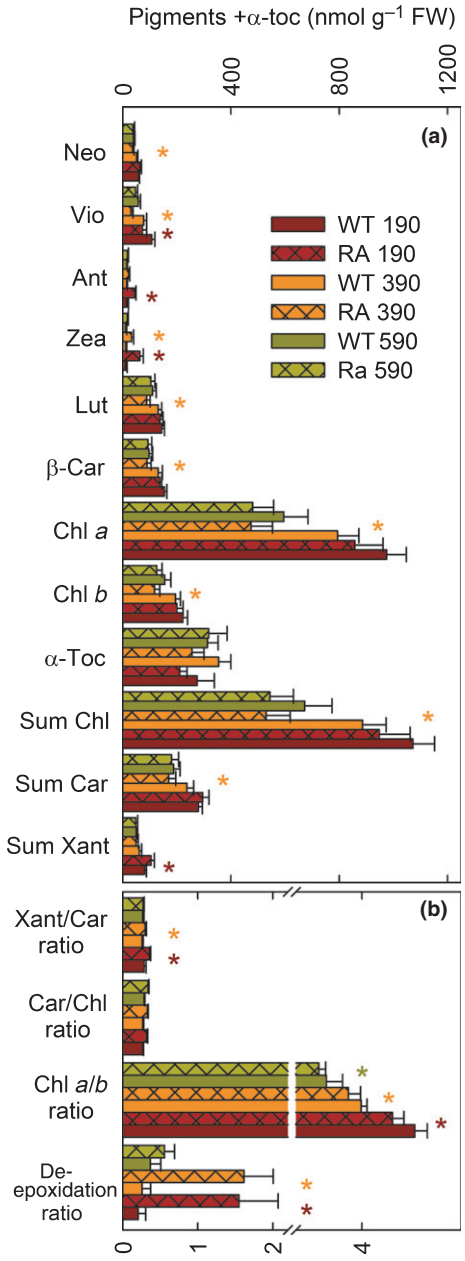

Fig. 5 (a) Photosynthetic pigment and α-tocopherol (α-toc) contents in isoprene-emitting (wild-type (WT)/control (C); solid bars) and isoprene emission suppressed plants (RA2/RA22; hatched bars) (red, 190 ppm; orange, 390 ppm; green, 590 ppm). Neo, neoxanthin; Vio, violaxanthin; Ant, antheraxanthin; Zea, zeaxanthin; Lut, lutein; β-Car, β-carotene; Chl *a*, chlorophyll *a*; Chl *b*, chlorophyll *b*; Sum Chl, chlorophyll *a* + chlorophyll *b*; Sum Car, 1β-carotene + violaxanthin + antheraxanthin + zeaxanthin + neoxanthin; Sum Xant, antheraxanthin + violaxanthin + zeaxanthin. (b) Ratios of: xanthophyll/β-carotene (Xant/Car); β-carotene/chlorophyll (Car/Chl); chlorophyll *a*/chlorophyll *b* (Chl *a*/*b*); de-epoxidation (zeaxanthin + 0.5×antheraxanthin)/(zeaxanthin + antheraxanthin + violaxanthin). Asterisks indicate significant differences between WT/C and RA2/RA22 within a CO_2 concentration (Tukey test; $P < 0.05$; mean ± SE; $n = 10$).

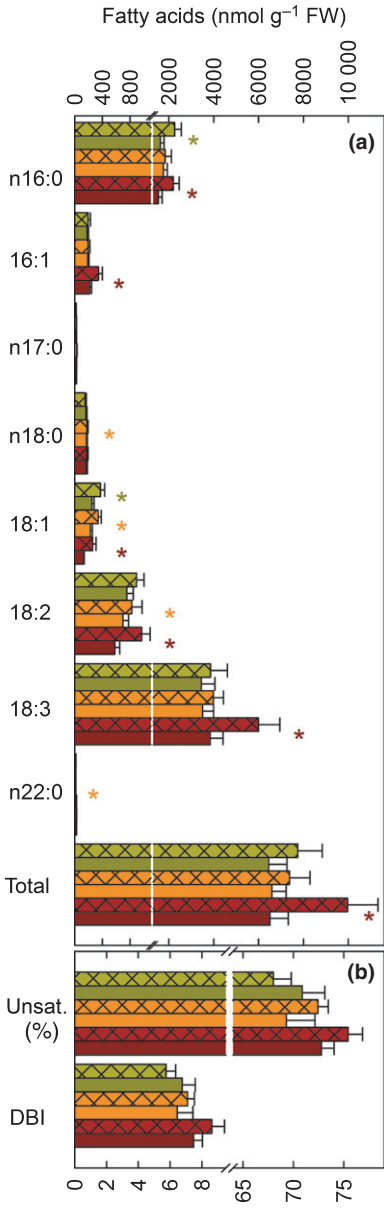

Fig. 6 Fatty acid content of the phospholipid fraction in C_{16}–C_{22} saturated (:0) and unsaturated (:1,:2,:3) compounds in leaves from both poplar isoprene-emitting lines (wild-type (WT)/control (C); solid bars) and isoprene emission suppressed lines (RA2/RA22; hatched bars) (red, 190 ppm; orange, 390 ppm; green, 590 ppm; n16:0, palmitic acid; 16:1, palmitoleic acid; n17:0, heptadecanoic acid; n18:0, stearic acid; 18:1, oleic acid; 18:2, α-linoleic acid; 18:3, α-linolenic acid; n22:0, behenic acid). The double bond index (DBI) was calculated using the following equation (Liljenberg & Kates, 1985): DBI = ((%16 : 1)+ (%18 : 1) + (2 × (%18 : 2)) +(3 × (%18 : 3))/((%n16 : 0) + (%n17 : 0) +(%n18 : 0) + (%n22 : 0)). Asterisks indicate significant differences between WT/C and RA2/RA22 within a CO_2 concentration (Tukey test; $P < 0.05$; mean ± SE; $n = 10$).

and possibly its isomer isopentenyl diphosphate (IDP), which might have affected the production of other MEP pathway products. In support of this, we found higher amounts of geranyl diphosphate (GDP) in suppressed lines, and up-regulation of another isoprenoid precursor, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (MEcDP). However, xanthophyll and carotenoid concentrations were lower in suppressed lines relative to WT/C. The inverse CO_2 dependences, whereby (1) concentrations of GDP and pigments are higher at low CO_2 and (2) the concentration of MEcDP is lower at low CO_2, indicate that different

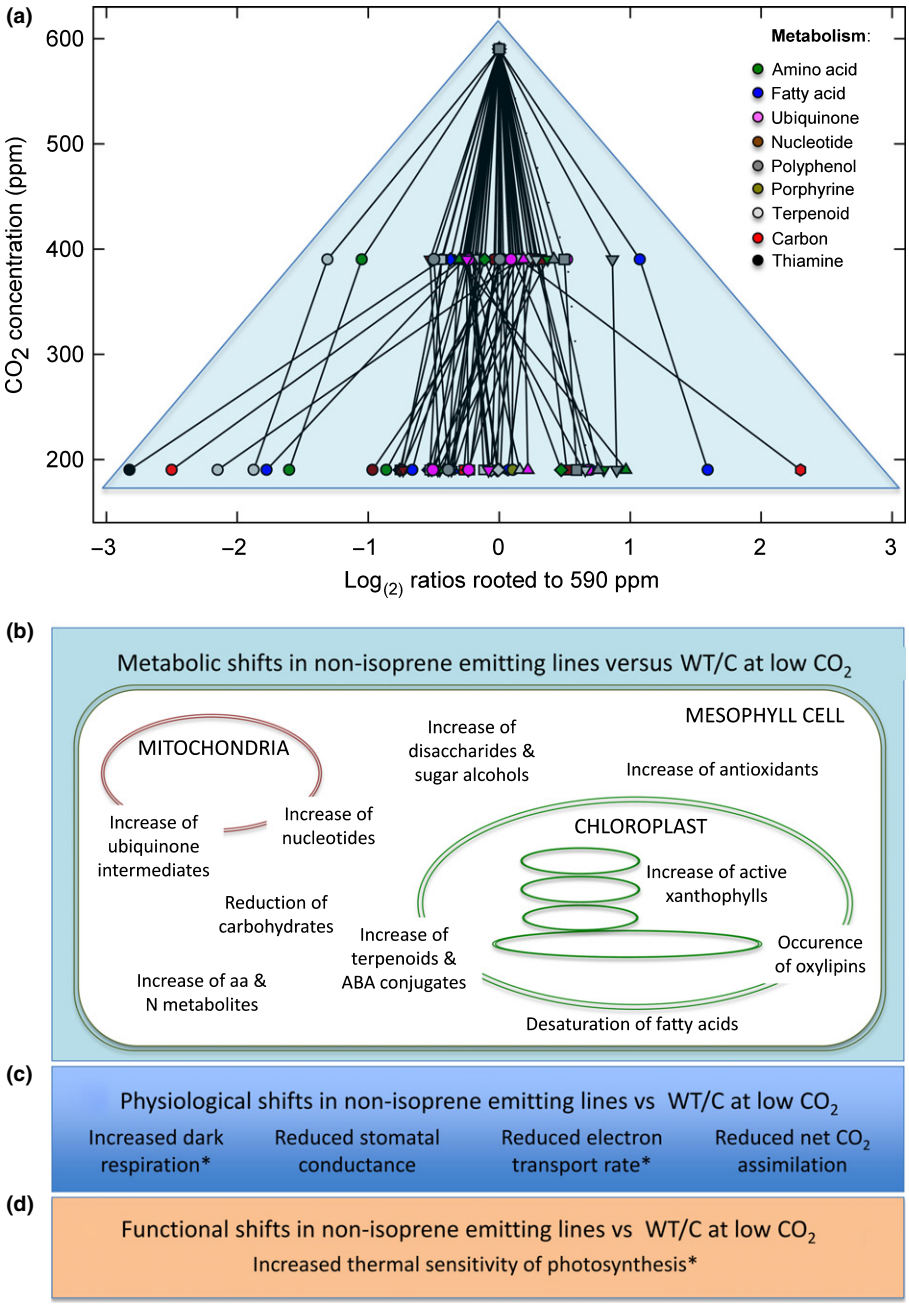

Fig. 7 (a) Metabolic differences between isoprene-emitting (wild-type (WT)/control (C)) and isoprene emission suppressed (RA2/RA22) poplar plants normalized to metabolic differences at 590 ppm. (b) Schema of metabolic shifts, and (c) their physiological and (d) functional impacts in isoprene emission suppressed lines compared with isoprene-emitting (WT/C) lines (*, see Way *et al.*, 2011). ABA, abscisic acid; aa, amino acids.

regulation mechanisms determine fluxes of metabolites within the MEP pathway and the subsequent pathway leading to higher terpenoids. Metabolic engineering of plastidic terpenoids impacts the formation of downstream products in transgenic *Arabidopsis thaliana* (Aharoni *et al.*, 2003) and *Mentha piperita* L. (Mahmoud & Croteau, 2001). Our previous studies at ambient CO_2 concentrations found that plastidic terpenoid concentrations were reduced (xanthophylls and carotenoids; Behnke *et al.*, 2007) or slightly increased (carotenoids; Behnke *et al.*, 2010b) in repressed lines compared with WT lines. The nontargeted metabolomic analysis showed that the impact of suppressing isoprene emission on terpenoid metabolism is generally smaller than expected, but is spread over different compounds, and has a strong CO_2 dependence. Analysis of the expression of different plastidic terpenoid genes (1-deoxy-D-xylulose 5-phosphate synthase (*DXS*), 1-deoxy-D-xylulose 5-phosphate reductoisomerase (*DXR*) and phytoene synthase (*PSY*)) across emitting and non-emitting lines at ambient CO_2 concentrations, however, showed no difference in transcript number throughout the growing season (Behnke *et al.*, 2010b). Transgenic lines also showed lower stomatal conductance, possibly as a result of higher amounts of abscisic acid glucose ester compared with WT/C: foliar ABA, which promotes stomatal closure, is derived from the MEP pathway (Barta & Loreto, 2006).

It seems, therefore, that the regulation of C flux toward the synthesis of isoprene and related metabolites is controlled at different biochemical and post-transcriptional levels (Guevara-Garcia *et al.*, 2005), all of which adjust terpenoid production. Earlier analysis of *DXS* activity, the putative controlling step of the MEP pathway (Lois *et al.*, 1998; Estévez *et al.*, 2001; Munoz-Bertomeu *et al.*, 2006; Vallabhaneni & Wurtzel, 2009), showed lower enzyme activities in repressed poplar lines compared with WT lines (Ghirardo *et al.*, 2010). Other regulatory controls might be under feedback mechanisms in the downstream part of the MEP pathway (Janowski *et al.*, 1996), such as DMADP accumulation (Wolfertz *et al.*, 2004). While the majority of our metabolic results show the negative CO_2 dependence we predicted, unsurprisingly some metabolites show other correlations with CO_2 that probably reflect alterations in metabolic fluxes or other processes not dealt with here. Consequently, future work should investigate the mechanisms controlling the regulation of the MEP pathway, as well as the effects of altering CO_2 on the individually affected compounds.

Alternate stress tolerance mechanisms are up-regulated in suppressed lines in a CO_2-dependent manner

The activity of *PcISPS:GFP* was regulated by growth CO_2 treatment, indicating a role for atmospheric CO_2 concentration as a signal for the transcriptional regulation of isoprene synthesis, along with other previously identified environmental factors such as light and temperature (Cinege *et al.*, 2009). Isoprene synthase appears to be localized to the chloroplasts (Wildermuth & Fall, 1996; Schnitzler *et al.*, 2005) and *PcISPS:GFP* activity was positively correlated with chlorophyll autofluorescence across all CO_2 concentrations and cell layers. Thus, isoprene promoter activity (and isoprene synthesis) was greatest where chlorophyll content, and thus photosynthetic capacity, were also highest. *ISPS* promoter activity is highest in palisade mesophyll cells (Cinege *et al.*, 2009), but the enhanced localization of *ISPS* promoter activity to this cell layer at our lowest CO_2 concentration implies a greater reliance on the physiological advantages provided by isoprene biosynthesis in these cells. While low-CO_2-grown plants also had the lowest net photosynthetic rates measured at their growth CO_2 (because of limited CO_2 substrate availability for photosynthesis), their high chlorophyll concentrations indicate that they would need to dissipate excitation energy toward alternative sinks, and thus support an enhanced need for stress tolerance mechanisms beyond that required for leaves that developed in higher CO_2 concentrations.

While reduced stress tolerance has been noted before in studies of the repressed lines grown at ambient CO_2 concentrations (Behnke *et al.*, 2007, 2010a,b), our results highlight the role of changes in growth CO_2 concentrations in exacerbating or diminishing stress-tolerance-related metabolic differences between isoprene-emitting and suppressed lines. Leaves from suppressed lines that developed at the lowest CO_2 concentration had the highest concentrations of antioxidants, and of thiamin monophosphate, a precursor of thiamin biosynthesis that has been linked to increased oxidative stress tolerance (Tunc-Özdemir *et al.*, 2009). Concentrations of de-epoxidized xanthophyll pigments were higher in suppressed lines grown at the lowest CO_2 concentration, compared with isoprene-emitting poplars, but this difference disappeared when trees were grown at the highest CO_2 concentration. A high de-epoxidation state is indicative of enhanced heat dissipation by nonphotochemical quenching in lines with suppressed isoprene emission (see Behnke *et al.*, 2007; Way *et al.*, 2011), and the de-epoxidation state increases in response to increases in the trans-thylakoid proton gradient that occur during insufficient dissipation of photosystem energy absorption (Holt *et al.*, 2004; Demmig-Adams & Adams, 2006). Atmospheric CO_2 concentrations also modulated the degree of unsaturation of fatty acids and the double bond index in the PLFAs, but only in non-isoprene-emitting lines. A shift toward unsaturation of the fatty acid component can provide stress tolerance to abiotic factors such as drought (e.g. Navari-Izzo *et al.*, 2006) and high temperature (e.g. Gombos *et al.*, 1994; Sato *et al.*, 1996; Burgos *et al.*, 2011), although it is often linked to low-temperature acclimation. These changes were accompanied by the occurrence of oxylipins when trees were grown at low CO_2 concentrations. As oxylipins can be produced by the oxygenation of polyunsaturated fatty acids by free radicals (Méne-Saffrané *et al.* 2008; Durand *et al.*, 2009), this may indicate increased oxidative damage in suppressed lines at the lowest CO_2 concentrations. The pronounced rise in phosphatidic acid concentrations in leaves of the RA lines grown at the lowest CO_2 concentration probably reflects a need to produce new membrane constituents under the higher stress conditions of a low-CO_2 environment, as phosphatidic acid is a precursor for the biosynthesis of many lipids (in particular acylglycerol lipids) and acts as a signaling lipid to stimulate fatty acid biosynthesis (Eastmond *et al.*, 2010; Hong *et al.*, 2010).

Lastly, while we focus on abiotic stress, isoprene production can also deter herbivory (Laothawornkitkul *et al.*, 2008). Jasmonic acid is important for up-regulating pathogen- and herbivore-defense pathways in leaves, and increased jasmonic acid concentrations in suppressed lines may reflect an increase in non-isoprene-mediated biotic defenses. Indeed, Behnke *et al.* (2012) observed a lower susceptibility of the same non-isoprene-emitting plants to the pathogenic fungus *Pollaccia radiosa*, compared with isoprene-emitting poplar lines.

Despite up-regulating multiple stress tolerance mechanisms, our earlier work showed that poplars with suppressed isoprene emissions still have a lower capacity for recovering from abiotic stress when grown at low CO_2 concentrations (Way *et al.*, 2011), demonstrating that this suite of changes does not fully compensate for the loss of isoprene biosynthesis. While the mechanism for isoprene-derived photosynthetic stress tolerance has been debated (Loreto & Schnitzler, 2010), in terms of whether it acts as an antioxidant (Vickers *et al.*, 2009) or stabilizes thylakoid membranes (Velikova *et al.*, 2011), our analysis shows that both antioxidant and membrane lipid metabolisms are significantly up-regulated when isoprene synthesis is suppressed, implying a role for both processes (see also Velikova *et al.*, 2012). As other isoprene-emitting species show similar changes in emission rates

with varying CO_2 (Wilkinson *et al.*, 2009), the conclusions drawn from our results may be broadly applicable. However, the extent to which any specific abiotic stress tolerance mechanisms are up-regulated by suppressing isoprene may depend on the extent to which a species relies on that mechanism.

We focus on the physiological and metabolic effects of suppressing isoprene biosynthesis under varying CO_2 concentrations, but these results have implications for larger evolutionary and ecological questions. It has been proposed that isoprene biosynthesis evolved in geological periods of low CO_2 (Way *et al.*, 2011; Monson *et al.*, 2013). Our findings are consistent with the potential for a strong selective pressure on isoprene biosynthesis at low CO_2. On an ecological scale, differences in isoprene emission can alter competitive relationships between species (Lerdau, 2007), favoring isoprene-emitting phenotypes in environments with frequent abiotic stress. Thus, convergence of the metabolic phenotypes of isoprene-emitting and non-isoprene-emitting species may alter community dynamics as atmospheric CO_2 concentrations rise, with significant implications for species composition in ecosystems such as tropical rain forests, which account for > 80% of global isoprene emissions (Müller *et al.*, 2008). Reduced biogenic isoprene emissions in a future climate also have implications for changes in atmospheric chemistry that impact climate forcing, including secondary organic aerosol formation rates (Kiendler-Scharr *et al.*, 2012) and atmospheric methane lifespans (Poisson *et al.*, 2000; Archibald *et al.*, 2011).

Conclusions

Differences in broad aspects of cellular metabolism between isoprene-emitting and non-isoprene-emitting plants diminished as the CO_2 concentration was increased. The compensatory increases in multiple stress tolerance pathways in non-emitting leaves emphasize the critical role of isoprene in stress tolerance when plants are grown at low CO_2 concentrations. As CO_2 concentrations continue to increase, the diminishing metabolic and physiological differences between leaves that do and do not emit isoprene imply that the benefit of producing isoprene for abiotic stress tolerance also diminishes, which could lead to reduced selection for the maintenance of isoprene synthase in a future high-CO_2 atmosphere.

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Supporting Information

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

Fig. S1 Chlorophyll autofluorescence and GFP fluorescence across cell layers in isoprene-emitting lines from varying growth CO_2 concentrations.

Fig. S2 (a) Venn diagram of metabolomic differences between isoprene-emitting (WT/C) and suppressed (RA2/RA22) poplars grown at different CO_2 concentrations analyzed by nontarget FTICR-MS, and (b) hierarchical cluster analysis of masses shown in the Venn diagram.

Table S1 Complete list of annotated masses sorted by metabolic pathways with the KEGG map, name of pathways, KEGG ID, aggregated names (as in Fig. 4) and possible isomers

Table S2 Results of two-way ANOVAs and Tukey tests for fatty acids and leaf pigments showing differences between WT/C and RA lines at different CO_2 concentrations

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