Leaf structural characteristics are less important than leaf chemical properties in determining the response of leaf mass per area and photosynthesis of *Eucalyptus saligna* to industrial-age changes in [CO₂] and temperature

Cheng-Yuan Xu¹²,*†, Anya Salih³, Oula Ghannour² and David T. Tissue⁴

¹ Department of Biological and Physical Sciences and Australian Centre of Sustainable Catchments, University of Southern Queensland, West Street, Toowoomba, QLD 4350, Australia
² CSIRO Ecosystem Sciences, EcoSciences Precinct, Boggo Road, Dutton Park, QLD 4102, Australia
³ Confocal Bio-Imaging Facility, School of Science and Health, University of Western Sydney, Richmond NSW 2753 Australia
⁴ Hawkesbury Institute for the Environment, University of Western Sydney, Richmond NSW 2753 Australia

* To whom correspondence should be addressed E-mail: cx2004@caa.columbia.edu
† Present address: Collaborative Research Network - USC Research Future Project, Environmental Futures Centre, Griffith University, Nathan QLD 4111, Australia.

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Abstract

The rise in atmospheric [CO₂] is associated with increasing air temperature. However, studies on plant responses to interactive effects of [CO₂] and temperature are limited, particularly for leaf structural attributes. In this study, *Eucalyptus saligna* plants were grown in sun-lit glasshouses differing in [CO₂] (290, 400, and 650 μmol mol⁻¹) and temperature (26 °C and 30 °C). Leaf anatomy and chloroplast parameters were assessed with three-dimensional confocal microscopy, and the interactive effects of [CO₂] and temperature were quantified. The relative influence of leaf structural attributes and chemical properties on the variation of leaf mass per area (LMA) and photosynthesis within these climate regimes was also determined. Leaf thickness and mesophyll size increased in higher [CO₂] but decreased at the warmer temperature; no treatment interaction was observed. In pre-industrial [CO₂], warming reduced chloroplast diameter without altering chloroplast number per cell, but the opposite pattern (reduced chloroplast number per cell and unchanged chloroplast diameter) was observed in both current and projected [CO₂]. The variation of LMA was primarily explained by total non-structural carbohydrate (TNC) concentration rather than leaf thickness. Leaf photosynthetic capacity (light- and [CO₂]-saturated rate at 28 °C) and light-saturated photosynthesis (under growth [CO₂] and temperature) were primarily determined by leaf nitrogen contents, while secondarily affected by chloroplast gas exchange surface area and chloroplast number per cell, respectively. In conclusion, leaf structural attributes are less important than TNC and nitrogen in affecting LMA and photosynthesis responses to the studied climate regimes, indicating that leaf structural attributes have limited capacity to adjust these functional traits in a changing climate.

Key words: Chloroplast, climate change, confocal microscopy, elevated [CO₂], global warming, leaf anatomy, leaf morphology, photosynthesis.

Introduction

Human activities have dramatically increased atmospheric concentrations of greenhouse gases since the industrial revolution. Due to fossil fuel combustion and land use changes, global atmospheric CO₂ concentration ([CO₂]) has risen from the
pre-industrial level of 280 µmol mol⁻¹ to ~390 µmol mol⁻¹ in less than two centuries, and may reach 600 µmol mol⁻¹ by the end of this century. Rising [CO₂] was accompanied by a temperature increase of 0.8 °C from 1850–1899 to 2000–2005, and an additional 1.8–4.0 °C warming (best estimations in different IPCC scenarios) is expected in this century (IPCC, 2007).

Clearly, rapid and large changes in [CO₂] and temperature may generate profound alterations in plant structure and function. The major knowledge gap in plant responses to climate change is that the interactive effects of [CO₂] and temperature have rarely been examined (Lloyd and Farquhar, 2008). In the limited studies that addressed this knowledge gap, most focused on plant growth and physiology, but did not investigate leaf structural attributes that are also known to contribute to the regulation of plant function (e.g. leaf anatomy, chlorenchyma cell size, chloroplast). Therefore, a more complete assessment of plant responses to rising [CO₂] and warming should include the role of plant structural attributes (Sallas et al., 2003). In addition, the majority of previous [CO₂] studies have compared modern [CO₂] and projected future [CO₂], but have not considered pre-industrial [CO₂] (but see Ghannoum et al., 2010a; b; Lewis et al., 2010; Logan et al., 2010; Tissue and Lewis, 2010; Ayub et al., 2011).

Leaf structural adaptation plays a central role in the overall adaptation of plants to changing atmosphere CO₂ (reviewed in Pritchard et al., 1999). In terms of leaf anatomy, elevated [CO₂] often generates greater leaf thickness and total mesophyll cross-sectional area, which are important determinants of photosynthetic rate. This phenomenon was mainly attributable to greater cell expansion (i.e. larger cell size) rather than enhanced cell division (i.e. more cells, Radoglou and Jarvis, 1992; Taylor et al., 1994; Ranasinghe and Taylor, 1996; Ferris et al., 2001) and may be driven by increased carbohydrate substrate availability (Pritchard et al., 1999). In leaf structural analyses conducted at different temperatures, most studies focused on plant responses to cold or heat stress, rather than non-stressful, warmer temperature conditions projected for this century (2–4 °C), and these results are generally inconsistent (Boese and Huner, 1990; Armstrong et al., 2006). Several studies conducted at a range of non-stressful growth temperatures (10–30 °C) found that for the same species, plants grown at higher temperatures had thinner leaves, which was mainly caused by reductions in the thickness of epidermal, palisade, and spongy layers, and an associated decrease in the size of mesophyll cells (Higuchi et al., 1999; Hartikainen et al., 2009; Gorsuch et al., 2010; Jin et al., 2011).

Alterations in plant chloroplast structure in elevated [CO₂] have been observed in different species (Griffin et al., 2001). In mature leaves, elevated [CO₂] increased chloroplast number per cell (Bockers et al., 1997; Wang et al., 2004; Teng et al., 2006) and/or the size of chloroplasts (Kutik et al., 1995; Robertson and Lecceh, 1995; Wang et al., 2004; Teng et al., 2006; Sinha et al., 2009), but the response pattern could vary due to the duration of exposure to [CO₂] and different leaf developmental stages (Robertson and Lecceh, 1995). In contrast, fewer studies have investigated the influence of warming on chloroplasts. In Arabidopsis thaliana, it was observed that the number of chloroplasts per cell remained unchanged under 2.5 °C warming, but decreased by 22% under 5 °C warming (relative to a control day/night temperature of 23/18 °C); meanwhile the size of chloroplasts also decreased with warmer growth temperature (Jin et al., 2011).

Leaf functional attributes are closely linked to their structure. It is widely accepted that leaf anatomy and mesophyll properties can affect carbon assimilation and leaf characteristics. Leaf thickness and mesophyll volume are strongly correlated with leaf area-based carbon assimilation (Higuchi et al., 1999; Niinemets et al., 2007) and leaf mass per area (LMA) (Gorsuch et al., 2010). It is also generally observed that there is a close correlation between rates of photosynthesis and chloroplast number (Ford and Shibles, 1988; Miroslavov and Kravkina, 1991; Jones et al., 1993). Higher chloroplast numbers accompanied by photosynthetic enhancement have been observed in plants grown under elevated [CO₂] (Wang et al., 2004) and ambient temperature relative to a warming treatment (Jin et al., 2011). However, these studies were conducted using two-dimensional (2D) imaging techniques, such as light or electron microscopy (i.e. chloroplast number was measured per cell cross-section or per unit leaf or cell cross-section area); subsequently, biological interpretation of the 3D organization of chloroplasts is complicated given that in practice, the formulation of the fundamental DeLesses’s principle may vary or need empirical correction case by case (Mayhew and Orive, 1974, 1975). In addition, few studies have combined leaf structural (e.g. anatomy and chloroplast parameters) and chemical attributes (e.g. nitrogen and carbohydrates) to address the determinants of functional traits (e.g. LMA and photosynthesis) (Smith et al., 2012). For example, it was found that despite strong positive correlations with Nₕₐₜₜ leaf functional properties.

In this study, laser confocal microscopy, which allowed both 2D and 3D assessments of structural changes, was applied to address the response of leaf anatomy and chloroplast parameters (number per cell and diameter) in Eucalyptus saligna (Sydney blue gum) to pre-industrial (290 µmol mol⁻¹), current (400 µmol mol⁻¹), or projected (650 µmol mol⁻¹) [CO₂] and to ambient or elevated temperature (ambient+4 °C). Eucalyptus saligna is a fast-growing local species adapted to the temperature, photoperiod, and soils of the Sydney region where the study was conducted. Its fast-growing nature made it likely to be strongly responsive to variation in climate variables and thereby suitable to be a model species. In addition, extensive research has been conducted regarding E. saligna’s growth and physiology (e.g. Barton et al., 2010; Ghannoum et al., 2010a; b; Logan et al., 2010; Ayub et al., 2011; Crous et al., 2011), which contributes to our understanding of physiological effects of leaf structural changes. In this study, the aim was to quantify responses of leaf anatomy and chloroplast parameters to industrial-age changes in atmospheric [CO₂] and temperature, and to examine whether responses of structural attributes could explain changes in leaf functional properties.
Interactive effect of [CO2] and temperature on leaf structural attributes

Materials and methods

Plant material

Seeds of Sydney blue gum (E. saligna Sm.) were obtained from Ensis (Australian Tree Seed Centre, ACT, Australia). Seedlings germinated at ambient [CO2] were transplanted into 10 litre cylindrical pots filled with 9 kg (air-dried mass) of loamy-sand soil for 4 weeks and located in six adjacent, naturally sun-lit glasshouse compartments, each of which had one of the six [CO2] and temperature treatment combinations. Thirty days after transplanting, seedlings were thinned to one seedling per pot. All plants were watered on a daily basis, and a commercial fertilizer solution (N: P: K: Ca: Mg: Fe: Mn: B 25:4:1:7:3:1:0:06:0:003:0:0.022%, at 0.2 g N l−1, General Purpose, Thrive Professional, Yates, NSW Australia) was applied on three occasions (30, 120, and 135 d after planting).

For the detailed experimental set-up, see Ghannoum et al. (2010a). In brief, three glasshouse compartments were maintained at current ambient local temperature and three glasshouse compartments were maintained at ambient+4 °C (higher temperature treatment). The average growing season temperatures for the ambient and high temperature treatments were 26/18 °C and 30/22 °C (day/night), respectively.

In brief, three glasshouse compartments were maintained at current, and projected [CO2] treatments, with average daytime [CO2] during the growth period at 290, 400, and 650 μmol mol−1, respectively (Ghannoum et al. 2010a).

Five seedlings of E. saligna were selected for each treatment and one visually mature leaf (the fifth leaf from the top of a branch, located in the middle of the canopy, fully expanded and with a well-developed cuticle layer) of each tree was used for analysis. Gas exchange measurements were made on selected leaves between 9:00h and 16:00h. Immediately following gas exchange measurements, the leaf was detached from the plant and cut into two pieces along the mid-vein (i.e. the vein was removed). These two leaf sections were used for the analysis of structural parameters and other leaf characteristics (LMA, photosynthetic rate, leaf nitrogen, and leaf carbohydrates, respectively).

Analysis of leaf, cell, and chloroplast parameters

For the confocal microscopic analysis, hand-cut cross sections of ~3 mm width were made at selected locations on the leaf (Fig. 1a). Prior to the analysis, sections were kept in distilled water for at least 20min. Smooth, well-levelled sections (one from each location) that avoided veins and oil glands were selected using a dissection light microscope (Leica MZ12, Leica Microsystems, Heidelberg, Germany) and mounted under a glass cover slip in distilled water on a glass slide for confocal microscopic imaging (Gomez-Casanovas et al., 2007). The aim was to use one section from each selected location of each sampled leaf (leaves n = 5 per treatment) for imaging. However, some leaf sections were found to be morphologically compromised and had to be discarded, and, therefore, the number of leaves and sections analysed were 3–5 leaves for each treatment and 2–4 sections per leaf. In total, confocal microscopy analysis was carried out on 87 sections from 24 leaves.

Confocal microscopy was performed using a Leica TCS SP5 confocal inverted microscope (Leica Microsystems) equipped with a HCX APO ×63 water immersion objective. The excitation wavelength was set at 488 nm laser line of an argon laser. Imaging of green emissions from the leaf cell walls was at 500–540 nm and of red chloroplast emissions (chlorophyll) at 650–700 nm using Leica SP5’s Acousto Optical Beam Splitter (AOBS®) for excitation–emission separation. The 3D images were obtained by performing 25–30 serial optical sections (1680 × 1680 pixels in the x–y plane, one pixel= ~0.147 μm) on the z-axis at 1 μm increments (Fig. 1b). Confocal imaging enabled the quantitative analysis of the 3D structure (see Supplementary Video S1 available at JXB online) of chloroplast parameters within a cell. Images were examined with IMARIS (Bitplane AG, Zurich, Switzerland) 3D analysis software.

A series of leaf, cell, and chloroplast parameters were determined from the 3D confocal images. The thickness of the upper epidermis, the palisade layer, the spongy layer, the lower epidermis, and the whole leaf was measured at multiple points (mostly five) of the section. The ratio of palisade layer to spongy layer was calculated accordingly. Five mesophyll cells that lay parallel to the section panel were selected from the palisade and spongy layers, respectively, and the cell size (length and width), the number of chloroplasts per cell, and the diameter of each chloroplast (distance between the two farthest points of a chloroplast) were quantified. The chloroplast gas exchange surface area (i.e. the total surface area of chloroplasts facing the intercellular space) of a mesophyll cell was calculated as the product of chloroplast number per cell and cross-sectional area of each chloroplast, assuming it to be in the shape of a circle. All length measurements were made with the software IMARIS (Bitplane AG) and ImageTool (University of Texas Health Science Centre, San Antonio, TX, USA). The number of cells observed was counted in three slices with an interval of 3 μm, for the palisade layer and the spongy layer in each section.

Photosynthetic capacity and leaf characteristics

Gas exchange measurements were conducted on attached leaves using a portable open gas exchange system (LI-6400, Licor, Lincoln, NE, USA) supplying photosynthetic photon flux density by an in-built red/blue light-emitting diode source. Leaf photosynthetic capacity was quantified by measuring the photosynthetic rate under saturating light (1200 μmol m−2 s−1) and [CO2] (1600 μmol mol−1) (Amax) (Ghannoum et al., 2010b). The leaf temperature was 28 °C and leaf-to-air vapour pressure deficit was 1.4–1.8 kPa. Leaf characteristics including LMA (g m−2), nitrogen content (Ng: g m−2), and carbohydrate content (soluble sugar, starch, and their sum total non-structural carbohydrates, TNCs). A half-leaf was measured for leaf area using a portable leaf area meter (LI-3100A, Li-Cor, freeze-dried, massed, then ground to a fine powder in a ball mill. Subsamples were analysed using a CN analyser (LECO TruSpec, LECO Cooperation, St. Joseph, MI, USA) for nitrogen content. Sugar, starch, and TNCs were measured following the protocol of Loveys et al. (2003). Photosynthetic capacity, leaf nitrogen, and carbohydrates are presented in both area-based and mass-based units (Amax: μmol m−2 s−1, N: mg m−2, S: mg m−2).
Statistical analysis

Data were analysed using a general linear model for a mixed-model factorial analysis of variance (ANOVA); [CO₂] and temperature were set as two fixed factors, and leaf, section, and cell were set as random factors with leaf nested in [CO₂]×temperature, section nested in leaf, and cell nested in section (Datadesk 6.0, Data Description Inc., Ithaca, NY, USA). The mean square value of leaf was used as an error term to test the main effect and the interaction of [CO₂] and temperature. Multiple comparisons among means were made with least significant difference.

To address whether responses in leaf structural attributes could explain changes in leaf function, multiple regression was performed following a stepwise regression procedure (StatPro, Indiana University, Bloomington, IN, USA) to determine the most important factors that affect LMA and Aₘₐₓ.

It was expected that any structural attribute that significantly explained the variation of Aₘₐₓ would be correlated with the photosynthetic product (TNCs), and, therefore, this prediction was tested with a linear regression. Moreover, linear regression was also used to examine the relationship between chloroplast number per cell and leaf light-saturated CO₂ assimilation rate at growth [CO₂] and temperature (Aₘₐₓ) with the data presented in Ghannoum et al. (2010b). These linear regressions were conducted with the software SMATR (Warton et al., 2006), and the slopes and interceptions were compared between various regression equations.

Results

Leaf anatomy and chloroplast parameters

The leaf thickness of E. saligna increased with rising [CO₂] and decreased with higher temperature (Fig. 2, Table 1a). About three-quarters of the leaf thickness change in response to [CO₂] and >88% of the change in response to warming was attributable to the response of the spongy layer [analysis of covariance (ANCOVA)]. The mean thickness of the palisade layer, the upper epidermis, and the lower epidermis showed similar response patterns to [CO₂] and temperature, but the magnitudes of their responses were smaller and the effects of [CO₂] and temperature were statistically insignificant in most cases; no [CO₂] by temperature interaction was observed for these parameters. In all treatments, a single layer of palisade cells was observed. On average, 23 palisade mesophyll cells and 32 spongy mesophyll cells were observed per section, showing no influence of [CO₂] or temperature (P=0.23–0.91, ANOVA).

Palisade and spongy mesophyll cells of E. saligna showed similar response patterns to [CO₂] and temperature with respect to cell size. Overall, the size of mesophyll cells of E. saligna increased with rising [CO₂] and decreased with higher temperature (Fig. 3, Table 1b). Across the pre-industrial to projected [CO₂] gradient, palisade and spongy mesophyll cells displayed significant increases in both cell length and width. In contrast, high temperature significantly decreased the width of palisade cells and the length of spongy cells. However, the shape of mesophyll cells (indicated by the length to width ratio) was not affected by [CO₂] or temperature treatment.

Rising [CO₂] and warming generated complex interactive effects on chloroplast number per cell and chloroplast diameter. In current and projected [CO₂], higher temperature reduced chloroplast number per cell, but did not change chloroplast diameter, and vice versa in pre-industrial [CO₂] (Fig. 4a–d). Palisade and spongy mesophyll cells generally showed similar response patterns, although the degree of statistical significance varied in response to temperature (Table 1b). In contrast, chloroplast gas exchange surface area per cell decreased with temperature, increased with [CO₂], and was not significantly affected by the interaction; however, the magnitude of the temperature response was larger in pre-industrial and current [CO₂] compared with projected [CO₂] (Fig. 4e–f, Table 1b).

Photosynthetic capacity and leaf characteristics

Leaf carbohydrates and LMA increased with rising [CO₂] but were not affected by growth temperature. Both elevated [CO₂] and warming reduced mass-based leaf nitrogen and Aₘₐₓ, while the [CO₂] effect was not significant, but the effect of [CO₂] was absent when nitrogen and Aₘₐₓ were converted to area-based units (Table 2). Leaf carbohydrates were affected by [CO₂] but not growth temperature. There were no significant interactive effects of [CO₂] and temperature on Aₘₐₓ or leaf characteristics.

Relationships between functional and structural traits

Seven variables ([CO₂], temperature, Sugararea, Starcharea, TNCarea, leaf thickness, and Narea) that might explain the variation in LMA were examined (with correlation among these variables shown in Supplementary Table S1a at JXB online). The optimum multiple regression equation suggested that 89% of the variation in LMA could be explained by TNCarea, leaf thickness, and temperature (Fig. 5). TNCarea was the most important factor that affected LMA, followed by leaf thickness, and then temperature (Table 3). Overall, TNCarea alone explains 80% of the variation of LMA (R²=0.8) as a statistical predictor, and its variation directly contributed to ~50% of the total variation of LMA in a physical sense (linear regression following Bertin and Gary, 1998; Bertin et al., 1999; Edwards et al., 2010; Fig. 1a).

To explore the determinants of Aₘₐₓ, seven variables ([CO₂], temperature, nitrogen, leaf thickness, chloroplast number per cell, chloroplast diameter, and chloroplast gas exchange surface area) were examined (see Supplementary Table S1b at JXB online for their correlations). The effect of LMA was adjusted by running the analysis with Aₘₐₓ-m and Nₘₐₓ, and the analysis was conducted twice, for palisade and spongy mesophyll chloroplast parameters, respectively. Stepwise regression suggested that Aₘₐₓ-m was primarily affected by Nₘₐₓ, followed by chloroplast gas exchange surface area, which is a structural measure of potential cellular gas exchange capacity. These two variables together explained ~63% of the variation in Aₘₐₓ-m (Fig. 6, Table 3).

As expected, chloroplast gas exchange surface area per cell was positively correlated with TNCarea. This relationship was valid for both palisade and spongy mesophyll cells and for each individual temperature treatment (Fig. 7); the slope of the regression lines was marginally significant (P=0.06, SMATR). Similar significant, positive correlations between chloroplast gas exchange surface area and TNC on a mass and nitrogen basis were also observed (data not shown).

Correlations between area- and mass-based Aₘₐₓ and the number of chloroplasts per cell were not significant (data not shown). However, chloroplast number per cell, for either palisade or spongy mesophyll, was positively correlated with Aₘₐₓ on a nitrogen basis (Fig. 8). In particular, the regression lines for palisade
Interactive effect of [CO2] and temperature on leaf structural attributes

Leaf thickness (µm)

<table>
<thead>
<tr>
<th>CO2 concentration</th>
<th>Temperature</th>
<th>Leaf thickness (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>290 µmol mol⁻¹</td>
<td>Ambient</td>
<td>32.2 (0.6)</td>
</tr>
<tr>
<td>400 µmol mol⁻¹</td>
<td>Ambient</td>
<td>54.0 (1.0)</td>
</tr>
<tr>
<td>650 µmol mol⁻¹</td>
<td>Ambient</td>
<td>55.6 (0.7)</td>
</tr>
<tr>
<td>290 µmol mol⁻¹</td>
<td>+4°C</td>
<td>33.0 (1.1)</td>
</tr>
<tr>
<td>400 µmol mol⁻¹</td>
<td>+4°C</td>
<td>52.3 (1.1)</td>
</tr>
<tr>
<td>650 µmol mol⁻¹</td>
<td>+4°C</td>
<td>48.6 (1.0)</td>
</tr>
</tbody>
</table>

Fig. 2. Effects of atmospheric [CO2] and growth temperature on leaf thickness of E. saligna, including the upper epidermis, palisade layer, spongy mesophyll layer, and lower epidermis. Stacked bars show mean (±SE) for layers and mean (+SE) for total leaf thickness. The percentages of total leaf thickness are marked for palisade and spongy mesophyll layers as mean (±SE).

Table 1. ANOVA results (P-values) of leaf structural traits measured in E. saligna grown at three atmospheric [CO2] and two air temperatures. Bold and italic fonts highlight significant (P < 0.05) and marginally significant (0.05 < P < 0.1) effects, respectively.

(a) The thickness of epidermis and mesophyll layers

<table>
<thead>
<tr>
<th>Source</th>
<th>Upper epidermis</th>
<th>Palisade layer</th>
<th>Spongy layer</th>
<th>Lower epidermis</th>
<th>Leaf thickness</th>
<th>Palisade/sponge</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO2</td>
<td>0.39</td>
<td>0.13</td>
<td>0.003</td>
<td>0.55</td>
<td>0.001</td>
<td>0.25</td>
</tr>
<tr>
<td>Temperature</td>
<td>0.66</td>
<td>0.94</td>
<td>0.004</td>
<td>0.004</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>C×T</td>
<td>0.11</td>
<td>0.23</td>
<td>0.80</td>
<td>0.35</td>
<td>0.29</td>
<td>0.48</td>
</tr>
</tbody>
</table>

(b) Mesophyll cell and chloroplast measures

<table>
<thead>
<tr>
<th>Source</th>
<th>Palisade mesophyll</th>
<th>Spongy mesophyll</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell length</td>
<td>Cell width</td>
</tr>
<tr>
<td>CO2</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>Temperature</td>
<td>0.99</td>
<td>0.02</td>
</tr>
<tr>
<td>C×T</td>
<td>0.20</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Fig. 3. Response of cell size (length and width) of palisade (a; filled symbols) and spongy mesophyll (b; x-marked symbols) to different [CO2] in ambient and +4 °C growth temperature. Values shown are the mean ±SE. Traits significantly affected by [CO2] or temperature are compared with least significant difference (LSD), and values followed by the same letter are not significantly different at the P=0.05 level.
and spongy mesophyll had a common slope \((P=0.95, \text{SMATR})\), despite significantly different intercepts \((P<0.001, \text{SMATR})\).

**Discussion**

In this study, leaf thickness and mesophyll cell size were found to increase with rising \([\text{CO}_2]\) and to decrease with warmer temperature. It was notable that there were no significant interactive effects of \([\text{CO}_2]\) and temperature on leaf anatomical parameters. In contrast, a significant interaction was observed in chloroplast parameters, which responded to warming with reduced chloroplast number per cell in pre-industrial \([\text{CO}_2]\), and decreased chloroplast diameter in current and projected \([\text{CO}_2]\). Consequently, chloroplast gas exchange surface area per cell, a parameter derived from chloroplast number per cell and chloroplast diameter and related to mesophyll cell gas exchange, increased with rising \([\text{CO}_2]\) and decreased with warming. It was also found that leaf structural attributes affected the variation of LMA and photosynthesis with the studied climate regimes, but their influences were less important than leaf chemical properties. In general, the response of LMA to \([\text{CO}_2]\) and temperature was primarily affected by the accumulation of TNCarea and secondarily by leaf thickness, while \(A_{\text{max}}\) and \(A_{\text{sat}}\) were primarily affected by leaf nitrogen and secondarily by chloroplast gas exchange surface area and chloroplast number per cell, respectively.

**Leaf anatomy**

Leaf anatomical responses of *E. saligna* to rising \([\text{CO}_2]\) and warming generally confirmed the pattern observed in the majority of previous studies, i.e. leaf thickness and mesophyll cross-sectional area increased with rising \([\text{CO}_2]\) but decreased with warming (Higuchi *et al.*, 1999; Pritchard *et al.*, 1999; Hartikainen *et al.*, 2009; Gorsuch *et al.*, 2010; Jin *et al.*, 2011). This pattern is congruent with the observation that higher
Table 2. The maximum photosynthetic capacity ($A_{\text{max}}$) and leaf characteristics of *E. saligna* grown at three atmospheric [CO$_2$] and two air temperatures with ANOVA results (P-values). Values shown are the mean (±SE). Means are compared with least significant difference (LSD), and values followed by the same letter are not significantly different at $P=0.05$. Bold and italic fonts highlight significant ($P < 0.05$) and marginally significant ($0.05 < P < 0.1$) effects, respectively.

Table 3. Multiple regression parameters. LMA was correlated to TNC$_{\text{area}}$, leaf thickness, and temperature. $A_{\text{max}}$ was correlated to $N_{\text{area}}$ and chloroplast gas exchange surface area, and two analyses were conducted for palisade and spongy mesophyll, respectively. Coefficients of independent variables and $R^2$ of the multiple regression equation, as well as their $P$-values are shown.
Fig. 5. Multiple linear regression result showing the relationship between leaf mass per area (LMA) and total non-structural carbohydrate (TNCarea), leaf thickness, and growth temperature. Relationships between LMA and TNCarea (a: $R^2=0.80$, $P<0.001$), the residual of LMA after fitting TNCarea and leaf thickness (b: $R^2=0.20$, $P=0.03$), and between the residual of LMA after fitting TNCarea and leaf thickness and temperature (c: $R^2=0.17$, $P=0.05$) are shown.

Fig. 6. Multiple linear regression results showing the relationship between maximum leaf photosynthetic capacity ($A_{\text{max}}$), leaf nitrogen content ($N_{\text{mass}}$), and chloroplast gas exchange surface area of mesophyll cells. Relationships between $A_{\text{max}}$ and $N_{\text{area}}$ (a: $R^2=0.50$, $P<0.001$) and between the residual of $A_{\text{max}}$ after fitting $N_{\text{area}}$ and chloroplast gas exchange surface area (b, palisade, filled symbols: $R^2=0.16$, $P=0.06$; c, spongy, x-marked symbols: $R^2=0.11$, $P=0.11$) are shown.
plant acclimation to high light (Oguchi et al., 2005, 2006; Niinemets et al., 2007). Second, since warming increases the chemical reaction rate of photosynthesis and accelerates CO₂ diffusion, a thinner leaf in a warmer climate would be sufficient to maintain a substantial carbon assimilation rate, while benefitting from reduced metabolic investment in leaf tissue. Finally, thinner leaves tend to have higher thermal conductivity, which increases energy loss by conduction across the leaf surface (Chandra, 2004), thereby improving leaf heat dissipation. This may be advantageous for maintaining an adequate thermal balance while reducing the demand for transpirational cooling at higher temperatures. These potential advantages may facilitate improved functioning of *E. saligna* in a warmer climate with higher [CO₂] in the future.

**Fig. 7.** Correlation between total non-structural carbohydrate (TNCarea) and chloroplast gas exchange surface area of palisade (a; filled symbols) and spongy (b; x-marked) mesophyll cells. Regression lines are shown (palisade: total, slope=0.019, R²=0.40, P < 0.001; ambient, slope=0.028, R²=0.49, P=0.01; +4 °C, slope=0.21, R²=0.63, P=0.001; spongy: total, slope=0.032, R²=0.59, P < 0.001; ambient, slope=0.054, R²=0.74, P < 0.001; +4 °C, slope=0.031, R²=0.81, P < 0.001).

**Fig. 8.** Relationships between nitrogen-based leaf CO₂ assimilation rate under light saturation (A_sat[^N]) and chloroplast number per cell for palisade (filled symbols) and spongy (x-marked symbols) mesophyll cells, respectively. Regression lines are shown (palisade: solid line, slope=0.96, R²=0.89, P=0.005; spongy: dotted line, slope=0.95, R²=0.95, P=0.001).

**Chloroplast parameters**

Chloroplast responses have been assessed in mature leaves under current and elevated [CO₂] in many different species (Griffin et al., 2001). Most studies observed an increased chloroplast number per cell and size in elevated [CO₂] (Kutik et al., 1995; Pritchard et al., 1997; Uprety et al., 2001; Wang et al., 2004; Teng et al., 2006). The size increment was attributable to increased cross-sectional width of the chloroplast (not quantified in this study) owing to a greater accumulation of starch grains, rather than chloroplast cross-sectional length (equivalent to diameter quantified in this study). In comparison, chloroplast parameters of *E. saligna* displayed similar responses to current to projected [CO₂], regardless of growth temperature, but the response to pre-industrial to current [CO₂] depended on the growth temperature. Studies on the response of chloroplasts to warming within a non-stressful temperature range (15–30 °C) are limited [e.g. *A. thaliana* displayed decreased chloroplast number per cell due to warming (day/night temperature 23/18 °C versus 28/23 °C) while chloroplast length did not change (Jin et al., 2011)]. This is also consistent with the observed temperature response of *E. saligna* in current and projected [CO₂]. However, the temperature response of *E. saligna* when grown in pre-industrial [CO₂] is different; high temperature did not affect chloroplast number per cell, but decreased chloroplast diameter. In summary, the present results confirm previously observed patterns of chloroplast parameters when grown in elevated [CO₂] and warming for *E. saligna* under the current to future climatic scenario, but indicate different temperature responses when grown in pre-industrial [CO₂].

Chloroplast gas exchange surface area (i.e. representing the total photosynthetic machinery in the mesophyll cell) increased with rising [CO₂] and decreased with warming in *E. saligna*, concomitantly with the dimensional change in leaf thickness and mesophyll cell size. For reasons discussed above, these responses may contribute to leaf acclimation to rising [CO₂] and growth temperature. In particular, the chloroplast gas exchange surface area per cell was adjusted downward in response to...
higher temperature. In *E. saligna*, this down-regulation in all \([\text{CO}_2]\) was achieved by reducing chloroplast diameter and decreasing chloroplast number, respectively. The mechanism by which these adjustments of chloroplast organization are achieved remains unknown (Bockers *et al.*, 1997). However, the acclimation significance of these two mechanisms may be related to the accumulation of starch grains in chloroplasts when grown in elevated \([\text{CO}_2]\) (Pritchard *et al.*, 1997; Wang *et al.*, 2004; Teng *et al.*, 2006). Starch accumulation may cause mechanical damage in chloroplasts and inhibit photosynthesis (Pritchard *et al.*, 1997); therefore, reducing chloroplast size may adversely affect carbon assimilation with rising \([\text{CO}_2]\). In contrast, in pre-industrial \([\text{CO}_2]\), where \([\text{CO}_2]\) is the primary factor limiting photosynthesis and starch overaccumulation is unlikely to occur, adjusting chloroplast diameter (and thus size) may be readily achievable because it does not involve more complex chloroplast biogenesis. Although not explicitly addressed in this study, this hypothesis may be tested by assessing alterations in the ultrastructure of chloroplasts in plants exposed to a range of pre-industrial to future \([\text{CO}_2]\) and temperature treatments. Notably, the magnitude of the response of chloroplast gas exchange surface area per cell to temperature was smaller in 650 \(\mu\text{mol mol}^{-1}\ [\text{CO}_2]\) than in 290 \(\mu\text{mol mol}^{-1}\ [\text{CO}_2]\) and 400 \(\mu\text{mol mol}^{-1}\ [\text{CO}_2]\), indicating that structural adjustment capacity may be approaching its limit.

**Correlation between leaf structural attributes and functional traits**

In broad-leaf plants, LMA is a product of leaf density and thickness. For *E. saligna*, the response of LMA to pre-industrial to future \([\text{CO}_2]\) and warming treatments was mainly the result of TNC accumulation (Bertin and Gary, 1998; Bertin *et al.*, 1999; Edwards *et al.*, 2010), which strongly affects leaf density (\(R^2=0.73, P < 0.0001\)), and altered leaf thickness, which has previously been observed (Pritchard *et al.*, 1997). It was found that changes in TNCarea were larger than changes in leaf thickness. Consequently, the dominant determinant of LMA was TNCarea (which explained 80% of the variation), while leaf thickness was secondary. This pattern suggests that leaf thickness in *E. saligna* may be constrained evolutionarily with limited capacity to influence LMA in a changing climate. It is notable that TNCarea and leaf thickness primarily explained the effect of \([\text{CO}_2]\) treatment on LMA, while a small, but significant, proportion of the variability of LMA was still attributable to the effect of warming. The observed positive effect of warming on LMA in *E. saligna* was in contrast to commonly observed negative relationships between LMA and higher growth temperature when expressed across biomes (Poorter *et al.*, 2009) or within species (Kao and Chang, 2001; Zhang *et al.*, 2005; Ogaya and Penuelas, 2007; Mendez-Alonzo *et al.*, 2008; Gorsuch *et al.*, 2010). The effect of leaf thickness on LMA has been largely described by the stepwise multiple regression procedure; therefore, the temperature–LMA correlation in *E. saligna* may mainly be attributable to altered leaf density. The constant number of mesophyll cells per cross-section in *E. saligna* suggests that the variation in cell density is limited. Therefore, the variability in LMA with higher temperature was more probably generated by variation in leaf structural chemical components (e.g. cell wall compounds such as lipids, structural carbohydrates, and lignin; Poorter *et al.*, 2009).

It is often observed that photosynthesis is positively correlated with chloroplast number (Ford and Shibles, 1988; Miroslavov and Kravkina, 1991; Jones *et al.*, 1993), but few studies have addressed this relationship with respect to rising \([\text{CO}_2]\) and temperature. Wang *et al.* (2004) found a proportional increase in chloroplast number per unit cell cross-sectional area and net photosynthesis per unit leaf area for tobacco (*Nicotiana sylvestris*) in elevated \([\text{CO}_2]\). Jin *et al.* (2011) observed similar concomitant changes in chloroplast number and photosynthesis for *A. thaliana* in different growth temperatures. In *E. saligna*, \(A_{\text{max}}\) and \(A_{\text{sat}}\) were affected by the pre-industrial to future \([\text{CO}_2]\) treatment, but not by higher temperature. These results suggest that the variation in \(A_{\text{max}}\) per leaf area was primarily determined by nitrogen content, and secondarily by chloroplast gas exchange surface area per mesophyll cell. Therefore, \(A_{\text{max}}\) was primarily regulated by the quantity of the photosynthetic apparatus, but secondarily by its spatial distribution, as indicated by significant correlations between chloroplast gas exchange surface area per cell and TNCarea (i.e. photosynthetic product). Similarly, it was found that chloroplast number per cell was positively correlated with \(A_{\text{sat}}\) per unit nitrogen, indicating that chloroplast number per cell in *E. saligna* was adjusted to optimize photosynthetic nitrogen use efficiency in growth conditions. In summary, the present results suggest that chloroplast parameters affected carbon assimilation of *E. saligna* in response to rising \([\text{CO}_2]\) and temperature, but that influence was limited; therefore, leaf nitrogen remained the primary factor affecting photosynthesis. Interestingly, in slow-growing *E. sideroxylon*, nitrogen also appeared to be a more important factor to influence \(A_{\text{max}}\) than the number of palisade layers (another structural attribute), despite a very different response pattern of \(A_{\text{max}}\). More studies are deserved to confirm whether this conclusion applies widely in more plant species.

In previous studies, the general relationship between chloroplast number and photosynthesis in response to climatic variables was established (Jin *et al.*, 2011; Wang *et al.*, 2004). However, there may be significant limitations associated with 2D leaf cross-section imaging (chloroplasts per cell cross-section or cross-section area) that hinder biological interpretation. The advantage of 3D imaging in plant structural and functional studies has been demonstrated in the literature (Armstrong *et al.*, 2006; Skaloud and Peksa, 2008; Chen and Liu, 2009; Omasa *et al.*, 2009; Wuyts *et al.*, 2010). For example, in an earlier study on *Opuntia ficus-indica*, the number of chloroplasts and mitochondria per cell was addressed, and a positive relationship between mitochondrial number per cell and leaf dark respiration rate was identified (Gomez-Casanovas *et al.*, 2007). Here, utilizing 3D confocal imaging in *E. saligna* further allows quantifying the number of chloroplasts within the whole cell. This advantage will facilitate scaling changes in cell chloroplasts to the leaf level and correlating these cellular changes with gas exchange properties, and thus may enhance identification of the structural adjustments that underpin functional changes in response to climate change.
Supplementary data

Supplementary data are available at JXB online.

Table S1. Correlations between LMA, Amax-m, and leaf chemical and structural attributes that may explain the relationship between LMA and Amax-m.

Video S1. Confocal imaging of the 3D structure of chloroplast parameters within a cell.

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