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Nitrogen in cell walls of sclerophyllous leaves accounts for little of the variation in photosynthetic nitrogen-use efficiency

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ABSTRACT

Photosynthetic rate per unit nitrogen generally declines as leaf mass per unit area (LMA) increases. To determine how much of this decline was associated with allocating a greater proportion of leaf nitrogen into cell wall material, we compared two groups of plants. The first group consisted of two species from each of eight genera, all of which were perennial evergreens growing in the Australian National Botanic Gardens (ANBG). The second group consisted of seven *Eucalyptus* species growing in a greenhouse. The percentage of leaf biomass in cell walls was independent of variation in LMA within any genus, but varied from 25 to 65% between genera. The nitrogen concentration of cell wall material was 0.4 times leaf nitrogen concentration for all species apart from *Eucalyptus*, which was 0.6 times leaf nitrogen concentration. Between 10 and 30% of leaf nitrogen was recovered in the cell wall fraction, but this was independent of LMA. No trade-off was observed between nitrogen associated with cell walls and the nitrogen allocated to ribulose 1·5-bisphosphate carboxylase/oxygenase (Rubisco). Variation in photosynthetic rate per unit nitrogen could not be explained by variation in cell wall nitrogen.

Key-words: cell wall nitrogen; leaf mass per unit area; nitrogen allocation; Rubisco; structural nitrogen.

INTRODUCTION

The photosynthetic capacity of a leaf is generally well-correlated with leaf nitrogen content. Although this relationship varies between species, much of the variation is related to another leaf parameter, specific leaf area (SLA), the projected leaf area per unit leaf dry mass. Thus, there exists a global function, regardless of life-form or location, which can predict photosynthetic capacity per unit leaf dry mass from nitrogen concentration and SLA (Reich, Walters & Ellsworth 1997; Wright *et al.* 2004). Photosynthetic rate per unit nitrogen [photosynthetic nitrogen-use efficiency (PNUE)] tends to decrease as SLA decreases (Poorter & Evans 1998; Hikosaka 2004). Because a smaller SLA is associated with greater leaf longevity, Field & Mooney (1986) suggested that there may be a trade-off between investing nitrogen in photosynthetic proteins such as ribulose 1·5-bisphosphate carboxylase/oxygenase (Rubisco) versus compounds required for longevity.

This hypothesis languished for lack of measurements of structural nitrogen in leaves. However, Onoda, Hikosaka & Hirose (2004) and Takashima, Hikosaka & Hirose (2004) developed methods for extracting detergent-soluble proteins from leaf material. They assumed that the nitrogen that remained behind represented cell wall protein. The comparison between evergreen and deciduous *Quercus* species (Takashima *et al.* 2004) revealed a clear trade-off between nitrogen invested in Rubisco and cell wall proteins. Leaves from evergreen *Quercus* had greater leaf mass per unit area (LMA, the reciprocal of SLA) and allocated a greater proportion of leaf nitrogen to cell wall protein than leaves from deciduous *Quercus*. Leaves of *Polygonum cuspidatum* also allocated a greater proportion of leaf nitrogen to cell walls as LMA increased (Onoda *et al.* 2004). However, for a given LMA, *Polygonum* allocated a smaller proportion of nitrogen to cell walls than *Quercus*. While both of these genera provide support for the hypothesis put forward by Field & Mooney (1986), the maximum LMA for leaves from both of these studies was only 60 g m^{-2}. This is at the lower end of the range reported by Reich *et al.* (1997), and so may not be representative of sclerophyllous, long-lived leaves.

Ellsworth *et al.* (2004) analysed leaf photosynthesis from 16 species with LMA ranging from 50 to 300 g m^{-2}. They calculated that the proportion of nitrogen allocated to Rubisco declined as LMA increased, and suggested that this was related to the need for greater investment in structural nitrogen. Clearly, there is a need for more data on cell wall nitrogen. Therefore, our first objective was to sample leaves from species representing a broad range of LMA to see whether the proportion of nitrogen allocated to cell walls was related to LMA. Two sampling strategies were used. Firstly, pairs of species from each of eight genera

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growing in the Australian National Botanic Gardens (ANBG) were chosen on the basis of contrasting LMA. These allowed phylogenetically independent contrasts to be made (Felsenstein 1985). Secondly, seven *Eucalyptus* species were grown and measured in a greenhouse to enable more comprehensive analyses of a single genus over a fourfold range in LMA.

Another feature of the relationship between PNUE and LMA is that for a given LMA, PNUE varies by an order of magnitude. It is likely that the nitrogen concentration in leaf structural biomass varies between species, and that this could account for some of the scatter. Therefore, our second objective was to assess how much of the variation in PNUE was associated with variation in the proportion of leaf nitrogen allocated to cell walls.

MATERIALS AND METHODS

Morphological and physiological measurements were obtained from two independent investigations: a field study comparing species pairs from eight genera and a greenhouse experiment that examined seven species of the genus *Eucalyptus*.

Plant material

A field study was conducted using two C_3 species from each of eight perennial Australian evergreen genera growing in the ANBG, Canberra (35°12″S, 149°04″E). The genera were selected so as to provide a wide range of LMA, and thus included a variety of growth forms including vines, shrubs and trees (Table 1).

The greenhouse study was conducted at the Australian National University, Canberra, using 12-month-old seedlings of *Eucalyptus bridgesiana*, *Eucalyptus elata*, *Eucalyptus mannifera*, *Eucalyptus moorei*, *Eucalyptus pauciflora*, *Eucalyptus polyanthemos* and *Eucalyptus rossii*, which were purchased from the Yarralumla Nursery (Canberra). At the nursery, seedlings were grown in potting mix that contained a controlled-release fertilizer (18% nitrogen), applied at a rate of 3 kg m^{-3}. All species were grown in full sunlight on the same site. Seedlings were transplanted from nursery tubes into large plastic pots (180 × 180 × 240 mm^3; length × width × depth) filled with a sterilized sand/peat/perlite mixture on 1 May 2006.

Growth conditions and experimental design of the greenhouse study

Five blocks containing 14 eucalypts were arranged in the greenhouse, with each block containing two replicates of each species. Greenhouses were maintained at 22–25 °C during the day and 15–18 °C at night. Supplementary lighting [280 μmol photosynthetically active radiation (PAR) photons m^{-2} s^{-1}] was provided by six 150 W flood lamps between 0500–1000 and 1700–2100 h in order to extend day length. Midday PAR measured with a quantum light sensor (Li-Cor Inc., Lincoln, NE, USA) averaged 500 μmol PAR photons m^{-2} s^{-1} on sunny days. Seedlings were watered to field capacity, twice daily. Rorison's nutrient solution (Hewitt 1966) was applied twice per week to each seedling, 100 mL from 1 May to 16 June, and 625 mL from 17 June to 1 July, to increase the size and growth rate of young leaves. For five of the replicates of each species, one per block, the 4 mM $Ca(NO_3)_2$ in the Rorison's solution was replaced with 4 mM $CaCl_2$, providing plus- and minus-nitrogen treatments, respectively. To distinguish between new and pre-existing leaves, white tags were attached to the youngest petiole on the main stem prior to the start of the experiment. Seedlings were periodically sprayed with chemicals for control of psyllids and powdery mildew.

Gas exchange measurements

Measurements of CO_2 assimilation rate per unit area (A_a) in the ANBG were made at saturating irradiance (Table 1), which was determined for each species by first measuring a light response curve. Measurements were made on leaves for all species except *Acacia*, where phyllodes were used, from 28 March 2006 to 24 April 2006, using an infrared gas analyser (IRGA) (LI-6400, Li-Cor) open gas exchange system. Where possible, a second LI-6400 was used on adjacent leaves of the same plant, allowing cross-checks for consistency in measurement. Steady-state measurements were made on similar fully expanded young leaves at an ambient CO_2 concentration (C_a) of 375 μmol mol^{-1}, between 0900 and 1500 h on sunny days. Leaf temperature was allowed to follow ambient conditions, which ranged between 15 and 32 °C.

Photosynthetic light response curves were measured for all seven greenhouse-grown eucalypt species. A standard irradiance of 1800 μmol PAR photons m^{-2} s^{-1} was adopted for all species during the measurement of CO_2 response curves, with the block temperature maintained at 22 °C, a flow rate of 500 μmol s^{-1} and the relative humidity of air entering the leaf chamber maintained between 70 and 80%. Following equilibration, A_a was measured at ambient CO_2 (375 μmol mol^{-1}) and a series of nine consecutive CO_2 concentrations from 50 to 1300 μmol mol^{-1}. As with the ANBG measurements, two LI-6400s were used.

At the time of measurement, only *E. elata*, *E. bridgesiana*, *E. mannifera* and *E. polyanthemos* plants had grown fully expanded, new leaves. There were no new leaves on minus-nitrogen *E. polyanthemos* plants at this time. To allow contrasts between all species, at least four pre-existing, non-necrotic leaves on different plants from the plus-nitrogen treatment were measured.

Scaling photosynthesis to a common C_i value

The model developed by Farquhar, von Caemmerer & Berry (1980) was used to scale all A_a measurements to a common intercellular CO_2 partial pressure (C_i) of 300 μmol mol^{-1} (A_{a300}). This approach was carried out using

Table 1. Leaf attributes of the congeners studied in the Australian National Botanic Gardens (ANBG)

Family	Genus	Species[1]	Growth form	LMA (g m^{-2})	N_m (mmol g^{-1})	A_a (μmol m^{-2} s^{-1})	PNUE [μmol (mol N)$^{-1}$ s^{-1}]	C_i/C_a	Δ (‰)
Fabaceae	*Acacia*	*beckleri*[b] Tindale	Shrub	212 ± 28*	1.71 ± 0.19 ns	9.1 ± 2.3 ns	50 ± 13 ns	0.52 ± 0.05*	22.3 ± 0.7**
		implexa[b] Benth.	Tree	126 ± 6	1.72 ± 0.05	9.1 ± 0.5	46 ± 3	0.72 ± 0.03	25.9 ± 0.3
Proteaceae	*Banksia*	*blechnifolia*[f] F. Muell.	Shrub	491 ± 17**	0.64 ± 0.04 ns	17.5 ± 4.1*	102 ± 18 ns	0.46 ± 0.05 ns	19.2 ± 0.4*
		serrata[a] Linn. f.	Tree	166 ± 6	0.54 ± 0.07	5.6 ± 1.0	137 ± 10	0.37 ± 0.02	20.7 ± 0.3
Myrtaceae	*Eucalyptus*	*pauciflora*[a] Sieber ex Spreng.	Tree	246 ± 10**	0.79 ± 0.04 ns	6.1 ± 0.5 ns	63 ± 6**	0.39 ± 0.01 ns	21.8 ± 0.2*
		radiata[a] DC.	Tree	114 ± 2	0.89 ± 0.02	6.3 ± 0.3	115 ± 10	0.43 ± 0.02	22.9 ± 0.2
Proteaceae	*Hakea*	*brownii*[a] Meisn.	Shrub	524 ± 12**	0.31 ± 0.03*	11.6 ± 1.3*	80 ± 7*	0.74 ± 0.03**	20.2 ± 0.2**
		salicifolia[a] (Vent.) B.L. Burtt	Shrub	129 ± 4	0.70 ± 0.08	7.5 ± 1.0	113 ± 11	0.59 ± 0.02	21.3 ± 0.1
Fabaceae	*Hardenbergia*	*comptoniana*[c] (Andrews) Benth.	Vine	75 ± 5**	2.41 ± 0.16**	16.0 ± 1.4*	123 ± 6 ns	0.61 ± 0.02*	22.0 ± 0.2*
		violacea[c] (Schneer.) Stearn	Vine	116 ± 5	1.38 ± 0.07	10.4 ± 0.8	104 ± 13	0.54 ± 0.01	20.2 ± 0.4
Byttneriaceae	*Lasiopetalum*	*discolor*[b] Hook.	Shrub	179 ± 15**	0.89 ± 0.01 ns	11.6 ± 0.7*	129 ± 8 ns	0.53 ± 0.03 ns	20.0 ± 0.3*
		schulzenii[a] (F. Muell.) Benth.	Shrub	87 ± 6	1.04 ± 0.06	6.7 ± 1.1	151 ± 13	0.42 ± 0.05	21.1 ± 0.3
Rhamnaceae	*Pomaderris*	*apetala*[d] Labill.	Shrub	81 ± 5**	0.77 ± 0.06 ns	7.3 ± 0.8 ns	144 ± 4 ns	0.66 ± 0.03 ns	22.5 ± 0.5*
		eriocephala[a] N.A. Wakef.	Shrub	196 ± 10	0.62 ± 0.05	7.2 ± 2.4	84 ± 25	0.53 ± 0.05	19.3 ± 0.5
Byttneriaceae	*Rulingia*	*magnifolia*[b] F. Muell.	Shrub	85 ± 4 ns	1.16 ± 0.03*	12.9 ± 0.3 ns	162 ± 7 ns	0.67 ± 0.01**	21.5 ± 0.3 ns
		salvifolia[b] Benth.	Shrub	78 ± 4	1.34 ± 0.06	12.8 ± 0.7	164 ± 7	0.61 ± 0.01	20.6 ± 0.5

Values are mean ± standard error for at least four oven-dried leaves.

[1]PPFD applied during the measurement of A_a: a, 1200; b, 1400; c, 1500; d, 1600; e, 1700; f, 1800 μmol PAR quanta m^{-2} s^{-1}.

N_m, leaf nitrogen concentration; A_a, CO_2 assimilation rate per unit area; PNUE, A_a normalised to C_i of 300 μmol mol^{-1} divided by leaf nitrogen content per unit area; C_i/C_a, ratio of intercellular to atmospheric CO_2; Δ, carbon isotope discrimination.

Asterisks indicate statistical significance for *t*-tests (two tailed and assuming unequal variance) for a given attribute within each genus, where $^{**}P < 0.001$, $^{*}P < 0.05$ and ns = not significant. (For additional parameters, see Supporting Information Table S1).

Eqn 1, assuming the *in vivo* maximum carboxylation activity of Rubisco per unit area (V_{cmax}) was limiting photosynthesis (A_a).

$$A_a = \frac{V_{cmax}(C_i - \Gamma_*)}{C_i + K_c(1 + O/K_o)} - R_d \quad (1)$$

The kinetic constants of Rubisco (K_c and K_o, the Michaelis–Menten constants for CO_2 and O_2, respectively, and Γ_*, the CO_2 compensation point in the absence of dark respiration, R_d) were adopted from von Caemmerer *et al.* (1994) assuming infinite internal conductance, with their temperature dependence functions given in von Caemmerer (2000). R_d was assumed to be 0.1 of A_a for the ANBG data. These assumptions were validated from CO_2 response curves measured on the eucalypt leaves (data not shown).

Morphological measurements

When gas exchange measurements were completed each day, leaves were detached and the segment used for measurement of photosynthesis was cut out and weighed. The segment area was determined using a leaf area meter (Li-Cor L3100, Li-Cor Inc.). Lamina thickness (T) was measured between the midrib and leaf edge with a Mitutoyo (Japan) analogue thickness gauge (precision ± 20 μm). T was calculated as the mean of four measurements. All ANBG leaf segments were dried for a minimum of 48 h at 80 °C then reweighed, allowing calculation of leaf dry mass per unit area (LMA). Water content (WC, mol m^{-2}) was computed as the change in leaf mass caused by drying, divided by leaf area. A duplicate set of leaves, matching those used for photosynthesis measurements, was sampled for cell wall nitrogen measurement, being snap-frozen in liquid nitrogen, then stored at –80 °C until used. Leaves were then freeze-dried at –45 °C, 64 mT for at least 3 d, using a Microprocessor Controlled Bench-Top Lyophilizer (FTS Systems, Inc., Stone Ridge, NY, USA). All sampled greenhouse eucalypt leaf segments were freeze-dried.

Leaf nitrogen measurements

Total leaf nitrogen

All dried leaves were ground separately in a ball mill. The nitrogen concentration of the photosynthetic segment was assayed using an elemental analyser (EA 1110 CHN-O; Carlo-Erba Instruments, Milan, Italy) with a typical machine precision of ±0.02% N. Approximately 1.2 mg of each segment was analysed.

Cell wall mass and nitrogen

A protocol was adapted from Lamport (1965) and Onoda *et al.* (2004) to remove soluble protein from the milled leaf material. Approximately 10 mg of freeze-dried leaf was extracted in 1.5 mL of buffer (50 mM tricine, pH 8.1) containing 1% PVP40 (average molecular weight 40 000, product no. 1407; Sigma Chemical Company, St Louis, MO, USA). The sample was vortexed, centrifuged at 12 000 *g* for 5 min (Eppendorf AG 5424, Hamburg, Germany) and the supernatant was carefully removed. The pellet was resuspended in buffer without PVP containing 1% sodium dodecyl sulphate (SDS), incubated at 90 °C for 5 min, then centrifuged at 12 000 *g* for 5 min. This was repeated, and then two washes with 0.2 M KOH, two washes with deionized water and finally two washes with ethanol were carried out. The tube containing the pellet was then oven-dried at 80 °C. The remaining dry mass of pellet was assumed to represent the leaf structural biomass, and the N content was determined on 2–5 mg of material using the elemental analyser as above.

The fraction of leaf nitrogen in cell wall material, N_{CW}/N_L, was calculated using Eqn 2:

$$\frac{N_{CW}}{N_L} = \frac{M_{CW}}{M_L} \times \frac{N_{CW}}{M_{CW}} \times \frac{M_L}{N_L} \quad (2)$$

where the fraction of cell wall material (M_{CW}) recovered from the total leaf biomass (M_L) was multiplied by the nitrogen concentration of cell wall material (N_{CW}/M_{CW}) divided by the leaf nitrogen concentration (N_L/M_L).

Attempts were made to extract Rubisco for several of the species using the method which has routinely been used for *Nicotiana tabacum* (Mate *et al.* 1993). We tried to grind fresh leaves, or leaves frozen in liquid N_2, using mortar and pestle, a Ten Broeck homogenizer or a Polytron, and we also tried to extract freeze-dried leaf material that was ball milled. None of our attempts yielded adequate soluble protein or Rubisco, presumably because we were unable to successfully rupture the mesophyll cells.

Calculation of Rubisco nitrogen and PNUE

The fraction of nitrogen allocated to Rubisco (N_R/N_L) was calculated from V_{cmax} (μmol CO_2 m^{-2} s^{-1}, derived from Eqn 1) as follows:

$$\frac{N_R}{N_L} = V_{cmax} \times \frac{M_R}{k_{cat}} \times \frac{[N_R]}{n_R} \div N_a \quad (3)$$

where M_R is the molecular mass of Rubisco, 0.55 g Rubisco (μmol Rubisco)$^{-1}$, k_{cat} is the catalytic turnover number at 25 °C, 3.5 mol CO_2 (mol Rubisco sites)$^{-1}$ s^{-1} (von Caemmerer *et al.* 1994), n_R is the number of catalytic sites per mole of Rubisco, 8 mol Rubisco sites (mol Rubisco)$^{-1}$ and [N_R] is the nitrogen concentration of Rubisco, 11.4 mmol N (g Rubisco)$^{-1}$ and N_a is the nitrogen content per unit leaf area (mmol N m^{-2}). This provides a minimum estimate as it assumes full Rubisco activation.

PNUE [μmol CO_2 (mol N)$^{-1}$ s^{-1}] was calculated by dividing the CO_2 assimilation rate per unit area scaled to a common C_i (A_{a300}), by the nitrogen content per unit leaf area (N_a).

We also inferred the fraction of leaf nitrogen allocated to Rubisco from the global relationship between photosynthetic rate per unit leaf nitrogen and LMA {PNUE [μmol CO_2 (mol N)$^{-1}$ s^{-1} = 587 × LMA (g m^{-2})$^{-0.435}$]} (Hikosaka 2004; Wright *et al.* 2004) using Eqn 4, as follows:

$$\frac{N_R}{N_L} = 587 \times LMA^{-0.435} \times \frac{V_{c\,max}}{A_{a250}} \times \frac{M_R}{k_{cat}} \times \frac{[N_R]}{n_R} \times 1.5 = LMA^{-0.435} \quad (4)$$

where from Eqn 1, a V_{cmax} of 100 μmol m^{-2} s^{-1} is required for an assimilation rate per unit leaf area, A_{a250} of 19.7 μmol m^{-2} s^{-1} when leaf temperature is 25 °C and intercellular CO_2 concentration is 250 μmol mol^{-1}. The factor 1.5 was necessary to scale the fit to published N_R/N_L data and may indicate that either the average C_3 species k_{cat} is only 2.33 mol CO_2 (mol Rubisco sites)$^{-1}$ s^{-1}, or Rubisco is not fully active.

Carbon isotope composition measurements

The carbon isotope composition (δ^{13}C) of leaf material sampled after gas exchange measurement was determined using an elemental analyser (EA 1110 CHN-O; Carlo-Erba Instruments) coupled to an isotope ratio mass spectrometer (VG Isochrom; Fisons Instruments, Manchester, UK). δ^{13}C values were obtained using approximately 0.1–0.2 mg of milled leaf. Typical machine precision was ±0.2‰ δ^{13}C. Composition values were converted to discrimination (Δ) values using Eqn 5 (Farquhar & Richards 1984):

$$\Delta = \left(\frac{\delta_a - \delta_p}{1 + \delta_p}\right) \times 1000‰ \quad (5)$$

where δ_a is the carbon isotope composition of the air (−8‰ = −8 × 10^{-3}), and δ_p is the measured carbon isotope composition of the plant material.

Statistical analysis

All statistics were determined using SPSS v12.0 (SPSS Australasia, Sydney, Australia). Student's *t*-tests were applied to determine whether a given attribute of the two species of each genus was significantly different. Regressions, linear or non-linear least square and associated P and adjusted R^2 values were calculated using the curve estimation function. All nominal variables (C_i/C_a, Δ, the fraction of leaf biomass in cell wall and the fractions of nitrogen allocated to cell walls or Rubisco) were arcsine square root transformed to meet the normality assumptions of parametric tests; however, graphs of these variables have been left untransformed for ease of interpretation. All figures were drawn with species means, including one standard error of the mean. Significant results are those with $P < 0.05$ unless otherwise stated.

RESULTS

Morphology, chemistry and physiology

LMA differed significantly between each species pair from the ANBG, with exception of the genus *Rulingia* (Table 1). There was a sevenfold variation in LMA across ANBG species (75–524 g m^{-2}, Table 1) and a fivefold variation across greenhouse eucalypts (50–240 g m^{-2}, Table 2). The greater spread of LMA with the ANBG species was primarily caused by the very high LMA values for *Banksia blechnifolia* and *Hakea brownii*. Similar variation was observed for total leaf nitrogen concentration (N_m), with approximately eightfold (0.3–2.4 mmol g^{-1}) and fourfold (0.6–2.5 mmol g^{-1}) ranges measured in the ANBG and greenhouse eucalypts, respectively. A threefold range in A_a was found in both surveys (5–18 μmol m^{-2} s^{-1}). Some species pairs had similar photosynthetic rates (e.g. *Acacia*), while others (e.g. *Banksia*) differed significantly. Leaf thickness was positively related to LMA [T (μm) = 1.6 × LMA (g m^{-2}) + 380, $R^2 = 0.44$, $n = 173$, $P < 0.001$ (slope) for the ANBG, T (μm) = 2.3 × LMA (g m^{-2}) + 92, $R^2 = 0.89$, $n = 71$, $P < 0.001$ (slope) for the greenhouse *Eucalyptus* leaves sampled from the ANBG followed the greenhouse regression and were generally thinner than other species for a given LMA]. WC of leaves was positively related to LMA [WC (mol m^{-2}) = 0.033 × LMA + 4.47, $R^2 = 0.83$, $n = 177$, $P < 0.001$ (slope) for the ANBG (with the exception of *Acacia beckleri* which had twice the WC (20.5 mol m^{-2}) for its LMA of 200 g m^{-2} compared to all the other species], WC (mol m^{-2}) = 0.068 × LMA + 3.5, $R^2 = 0.88$, $n = 72$, $P < 0.001$ (slope) for the greenhouse data. The WC of leaves from the greenhouse *Eucalyptus* was about 50% more than for leaves from other genera from the ANBG for any given LMA with the exception of *A. beckleri*. The ratio of CO_2 concentration in the intercellular space (C_i) to that in the IRGA cuvette (C_a) was smaller and more variable for the ANBG species (0.37–0.74; Table 1) than the greenhouse *Eucalyptus* (0.78–0.88; Table 2). The relationship between Δ and C_i/C_a deviated from the expected relationship for the ANBG data (Table 1). Small C_i/C_a ratios were not associated with small Δ values, possibly reflecting the dry conditions during the gas exchange survey. For the greenhouse *Eucalyptus*, Δ values were consistent with the measured C_i/C_a, and although the correlation was not strong ($R^2 = 0.17$), it was significant (slope $P = 0.005$, $n = 41$; Table 2). The nitrogen treatment applied to the *Eucalyptus* plants significantly increased nitrogen concentration per unit leaf dry mass and increased photosynthetic rate, both per unit leaf area and per unit leaf nitrogen.

Relationships between cell wall biomass, cell wall nitrogen and LMA

The fraction of leaf biomass recovered as cell walls was independent of LMA within *Eucalyptus* and ANBG genera (Fig. 1). In general, ANBG species had greater proportions of biomass in cell walls. The highly sclerophyllous genus *Banksia* had around 0.65 of leaf biomass in cell walls compared to 0.30 for *Eucalyptus*. For *Eucalyptus* leaves, the proportion of leaf biomass in cell walls for a given LMA was not affected by leaf age, N treatment or whether the plants were grown in the ANBG or greenhouse.

Table 2. Leaf attributes of *Eucalyptus* species grown in a greenhouse (see Methods)

Species	Old/Young	N treatment	LMA (g m^{-2})	N_m (mmol g^{-1})	A_a (μmol m^{-2} s^{-1})	PNUE [μmol (mol N)$^{-1}$ s^{-1}]	C_i/C_a	Δ (‰)
Eucalyptus bridgesiana[a]	O	–N	80.8 ± 3.1	1.13 ± 0.10	11.1 ± 1.8	119.1 ± 14.2	0.82 ± 0.02	24.6 ± 0.4
E. bridgesiana[a]	Y	–N	54.5 ± 2.8	1.32 ± 0.10	9.0 ± 0.7	125.2 ± 7.0	0.86 ± 0.02	25.2 ± 0.3
E. bridgesiana[a]	Y	+N	49.5 ± 2.4	1.85 ± 0.05	13.7 ± 1.3	153.3 ± 12.9	0.84 ± 0.02	24.6 ± 0.2
Eucalyptus elata[b]	O	–N	63.3 ± 5.5	0.72 ± 0.04	5.1 ± 0.4	111.4 ± 4.9	0.88 ± 0.01	22.6 ± 0.8
E. elata[b]	Y	+N	57.0 ± 2.3	1.02 ± 0.02	10.2 ± 0.8	179.2 ± 10.9	0.86 ± 0.02	26.2 ± 0.2
Eucalyptus mannifera[c]	O	–N	94.8 ± 10.4	0.90 ± 0.09	15.9 ± 2.9	172.5 ± 15.5	0.86 ± 0.02	25.6 ± 0.8
E. mannifera[c]	Y	–N	62.2 ± 3.1	1.21 ± 0.13	9.6 ± 1.3	125.2 ± 11.4	0.91 ± 0.02	26.0 ± 0.1
E. mannifera[c]	Y	+N	57.5 ± 11.7	1.41 ± 0.17	12.6 ± 1.8	162.2 ± 8.8	0.81 ± 0.05	25.6 ± 0.5
Eucalyptus moorei[d]	O	+N	241.0 ± 10.1	0.59 ± 0.05	15.7 ± 0.3	110.0 ± 9.2	0.78 ± 0.02	21.6 ± 0.4
Eucalyptus pauciflora[e]	O	+N	172.9 ± 6.9	0.64 ± 0.06	16.3 ± 1.3	140.1 ± 9.7	0.78 ± 0.03	23.7 ± 0.4
Eucalyptus polyanthemos[f]	O	+N	121.5 ± 6.7	1.14 ± 0.07	16.4 ± 0.6	117.0 ± 5.8	0.86 ± 0.02	23.5 ± 0.3
E. polyanthemos[f]	Y	+N	53.0 ± 3.2	2.51 ± 0.32	17.7 ± 1.8	138.9 ± 7.0	0.85 ± 0.02	24.4 ± 0.1
E. rossii[g]	O	+N	181.4 ± 8.3	0.69 ± 0.08	18.3 ± 1.3	145.4 ± 7.4	0.82 ± 0.02	23.7 ± 0.9

[a]R.T. Baker, [b]Denh., [c]A. Cunn. ex Benth., [d]Maiden & Cambage, [e]Sieber ex Spreng., [f]Schau., [g]R.T. Baker & H.G. Sm.

Abbreviations: N_m, leaf nitrogen concentration; A_a, CO_2 assimilation rate per unit area; PNUE, A_a normalised to C_i of 300 μmol mol^{-1} divided by leaf nitrogen content per unit area; C_i/C_a, ratio of intercellular to atmospheric CO_2; and Δ, carbon isotope discrimination.

Values for LMA, N_m, A_a and C_i/C_a are mean ± standard error of at least four leaves; values for Δ are mean ± standard error of three leaves. For all species, A_a values were measured at a PPFD of 1800 μmol quanta m^{-2} s^{-1}.

All leaves were freeze-dried after photosynthetic measurements (see Supporting Information Table S1).

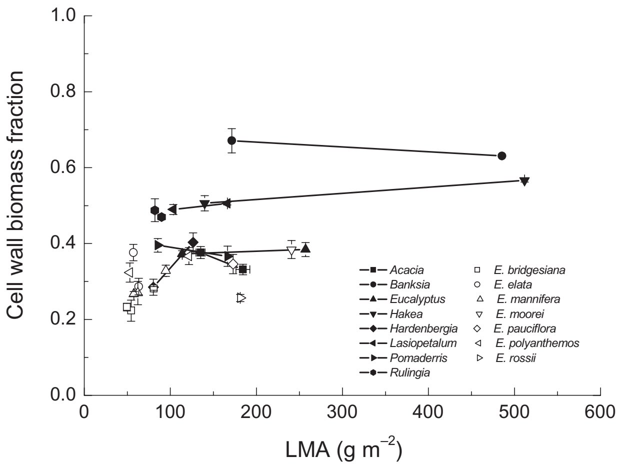

Figure 1. The fraction of leaf biomass recovered in cell walls as a function of leaf mass per unit area (LMA). Australian National Botanic Gardens (ANBG) species pairs have solid symbols joined by solid lines. *Eucalyptus* species grown in the greenhouse have hollow symbols. Error bars denote one standard error of the mean.

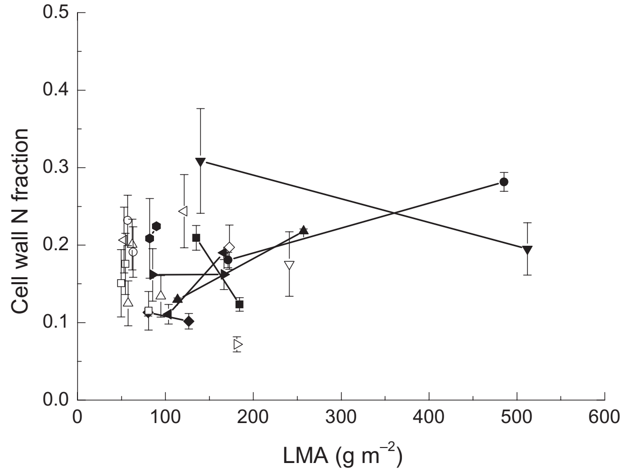

Figure 3. The fraction of leaf nitrogen present in cell walls as a function of leaf mass per unit area (LMA). Australian National Botanic Gardens (ANBG) species pairs have solid symbols joined by solid lines; *Eucalyptus* species grown in the greenhouse have hollow symbols, as defined in Fig. 1.

The nitrogen concentration of cell wall material was roughly 0.4 times leaf nitrogen concentration for all the species sampled from the ANBG, and 0.6 times leaf nitrogen concentration for *Eucalyptus* (Fig. 2). For *Eucalyptus*, the two species sampled from the ANBG fell within the greenhouse data. Leaves from the leguminous species (*Acacia* and *Hardenbergia*) had noticeably higher leaf nitrogen concentrations than did the non-leguminous species.

The fraction of leaf nitrogen recovered in cell walls was independent of LMA, both within and across genera (Fig. 3). The fraction of nitrogen allocated to cell walls for the ANBG *Eucalyptus* species fell within the range observed for the greenhouse-grown *Eucalyptus* species. Although there was an increase in the fraction of nitrogen associated with cell walls with increasing LMA between the two ANBG *Eucalyptus* species, the difference was not significant. Indeed, there was no overall tendency for an increasing proportion of leaf nitrogen to be allocated to cell walls with increasing LMA in the greenhouse *Eucalyptus*. The only other significant increase in the fraction of cell wall nitrogen with increasing LMA in the ANBG species was for the genus *Lasiopetalum*. There was a threefold spread (0.1–0.3) in the fraction of nitrogen recovered in cell walls for leaves with an LMA of 150 g m^{-2}.

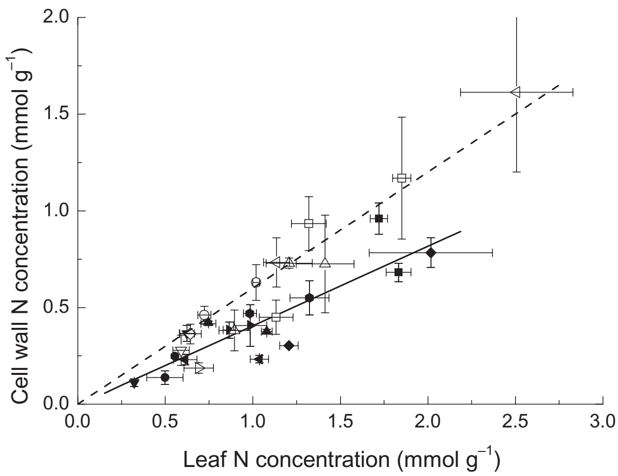

Figure 2. Cell wall nitrogen concentration versus leaf nitrogen concentration. Australian National Botanic Gardens (ANBG) species (solid symbols as shown in Fig. 1), regression [N_{CW}] = 0.41 [N_L], $R^2 = 0.76$, $n = 16$, $P < 0.001$. *Eucalyptus* species grown in the greenhouse (hollow symbols as shown in Fig. 1), regression [N_{CW}] = 0.59 [N_L], $R^2 = 0.90$, $n = 13$, $P < 0.001$.

Relationship between PNUE and LMA

PNUE calculated with a C_i value of 300 μmol mol^{-1} was weakly but negatively associated with LMA for the ANBG species [PNUE = 131 ($\pm$7) − 0.12 ($\pm$0.03) × LMA, $R^2 = 0.12$, $n = 93$, slope $P < 0.001$, Fig. 4]. A similar relationship was apparent for *Eucalyptus*, although this relationship was not significant [PNUE = 145 ($\pm$7) − 0.08 ($\pm$0.06) × LMA, $R^2 = 0.70$, $n = 71$, slope $P = 0.2$]. Indeed, the variation apparent in PNUE for a given greenhouse *Eucalyptus* LMA was nearly as great as that across the 10-fold range in LMA of the ANBG species. The *Acacia* species had the lowest PNUE values, whereas *Banksia* species had among the highest despite having the greatest fraction of leaf biomass recovered in cell walls. No general relationship existed between PNUE and the fraction of nitrogen in cell walls (Fig. 5). *Eucalyptus* was the only ANBG genus that had significantly reduced PNUE associated with greater fraction of nitrogen in cell walls, and while a negative relationship was also observed for the greenhouse *Eucalyptus*

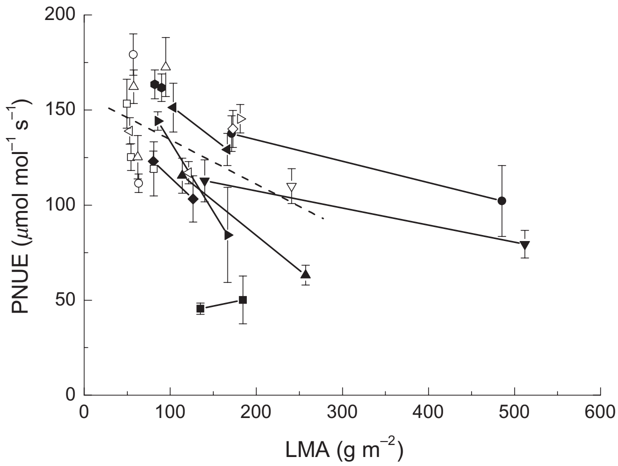

Figure 4. Relationship between photosynthetic nitrogen-use efficiency (PNUE) and leaf mass per unit area (LMA). Photosynthesis was measured at 1200–1800 μmol PAR photons m^{-2} s^{-1} for Australian National Botanic Gardens (ANBG) genera and 1800 μmol PAR photons m^{-2} s^{-1} for the greenhouse species, then adjusted to a common C_i of 300 μmol mol^{-1}. ANBG species pairs have solid symbols joined by solid lines; *Eucalyptus* species grown in the greenhouse have hollow symbols, as defined in Fig. 1. The regression for *Eucalyptus* (dashed line) was PNUE = 157.7 (±12) − 0.234 (±0.095) LMA, $r^2 = 0.31$, $n = 15$.

[PNUE = 155 (±9) − 70 (±29) × [N_{CW}/N_L], $n = 61$, slope $P = 0.02$], it was very weak ($R^2 = 0.08$). Therefore, the decline in PNUE as LMA increases is not associated with an increased fraction of nitrogen in the cell walls.

Relationships between the fractions of leaf nitrogen in Rubisco or cell walls

As we were unable to extract Rubisco from the leaves, we calculated the fraction of nitrogen in Rubisco, assuming a

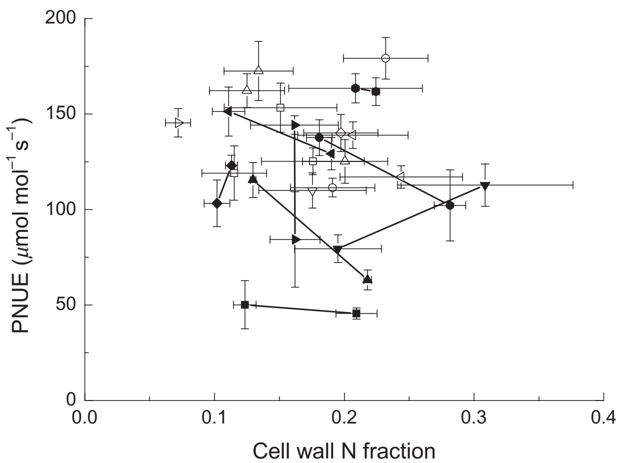

Figure 5. Relationship between photosynthetic nitrogen-use efficiency (PNUE) and the fraction of leaf nitrogen present in cell walls. Australian National Botanic Gardens (ANBG) species pairs have solid symbols joined by solid lines; *Eucalyptus* species grown in the greenhouse have hollow symbols, as defined in Fig. 1.

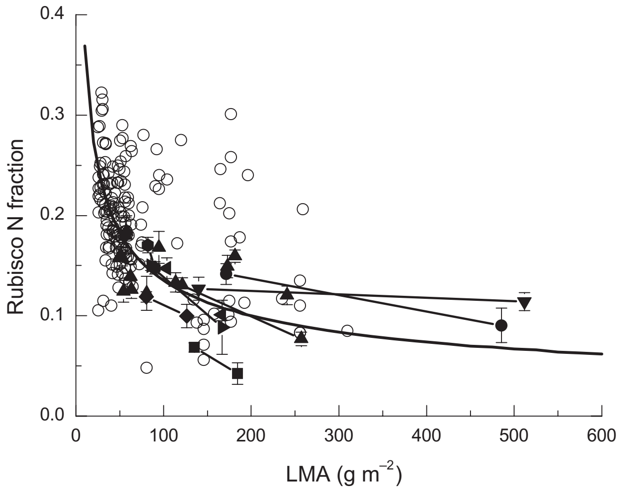

Figure 6. The fraction of leaf nitrogen in Rubisco in relation to leaf mass per unit area (LMA). Australian National Botanic Gardens (ANBG) species pairs have solid symbols joined by solid lines (as defined in Fig. 1); *Eucalyptus* species grown in the greenhouse (▲). Published data where Rubisco has been assayed biochemically (○): Ethier *et al.* (2006) (*Pseudotsuga menziesii*), Evans *et al.* (1994) (*Nicotiana tabacum*), Evans & Seemann (1984) (*Triticum aestivum*), Gleadow *et al.* (1998) (*Eucalyptus cladocalyx*), Hikosaka *et al.* (1998) (*Quercus mysinaefolia*, *Chenopodium album*), Katahata *et al.* (2007a,b) (*Daphniphyllum humile*), Kuppers (1996) (*Acacia* and *Eucalyptus* spp.), Onoda *et al.* (2004) (*Polygonum cuspidatum*), Poorter & Evans (1998) 10 species, Takashima *et al.* (2004) (*Quercus* spp.), Warren, Adams & Chen (2000) 10 species. The solid curve is calculated from the power relationship between PNUE and LMA (Hikosaka 2004; Wright *et al.* 2004), $N_R/N_L = LMA^{-0.435}$, see methods.

constant set of kinetic parameters for all species (Eqn 3). The fraction of nitrogen in Rubisco decreased as LMA increased for each of the eight species pairs sampled from the ANBG [[N_R/N_L] = 0.13 (±0.01) − 9.4 × 10^{-5} (±3.1 × 10^{-5}) × LMA, $R^2 = 0.08$, $n = 92$, slope $P = 0.003$, Fig. 6]. There was no equivalent relationship evident for the *Eucalyptus* data. However, published data from many other species where Rubisco protein content has been directly measured make the overall inverse relationship as a function of LMA more obvious (see the hollow circles in Fig. 6). The solid curve is calculated from the relationship between PNUE and LMA in the Glopnet database (Wright *et al.* 2004); see Eqn 4. There is broad overlap between values based on Rubisco protein assays and those calculated from gas exchange measurements. Overall, the fraction of nitrogen in Rubisco increases rapidly as LMA is reduced below 100 g m^{-2} and decreases more gradually as LMA increases above 100 g m^{-2}.

By calculating the nitrogen cost of Rubisco, it is possible to directly compare any trade-off between nitrogen allocated to Rubisco versus cell walls (Fig. 7). Nitrogen allocation to Rubisco significantly decreased as more nitrogen was allocated to cell walls for only three of the species pairs (*Banksia*, *Lasiopetalum*, *Eucalyptus*). Out of these, cell wall nitrogen allocation only differed significantly between the two *Lasiopetalum* species. Moreover, there was

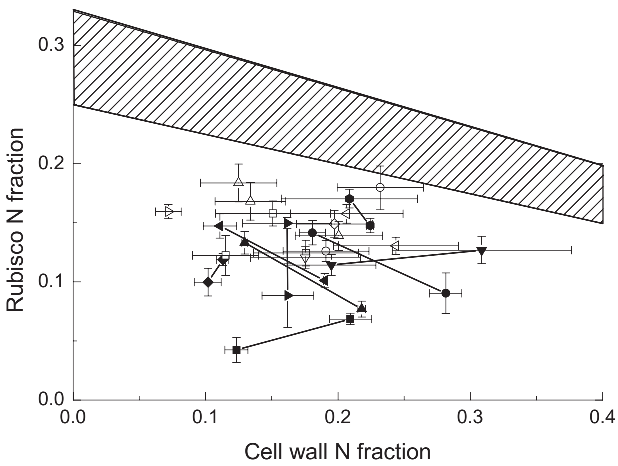

Figure 7. The fraction of leaf nitrogen in ribulose 1·5-bisphosphate carboxylase/oxygenase (Rubisco) in relation to the fraction of leaf nitrogen in cell walls. Nitrogen in Rubisco was calculated from measurements of photosynthesis (see Methods). Australian National Botanic Gardens (ANBG) species pairs have solid symbols joined by solid lines, and *Eucalyptus* species grown in the greenhouse have hollow symbols, as defined in Fig. 1. The shaded zone represents the upper bound assuming that on average, Rubisco represents one quarter to one-third of the nitrogen in soluble and thylakoid protein (Evans & Poorter 2001).

no significant relationship between nitrogen allocated to Rubisco and that to cell walls for the eight *Eucalyptus* species measured in the greenhouse study. The fraction of nitrogen allocated to Rubisco in *E. pauciflora* was much lower in leaves sampled from the ANBG (0.08) than from the greenhouse (0.15), but similar fractions of nitrogen were recovered in their cell walls (0.2). The only significant increase in both the fraction of nitrogen in Rubisco and in cell walls was for the *Acacia* species pair. Overall, these data did not suggest that an increased allocation of nitrogen to cell walls occurred at the expense of nitrogen allocation to Rubisco.

DISCUSSION

Consistent with expectations based on worldwide observations (Reich *et al.* 1997; Hikosaka 2004; Wright *et al.* 2004), the rate of photosynthesis per unit leaf nitrogen, PNUE, was negatively related to LMA for the ANBG leaves sampled in this study (Fig. 4). The negative correlation was also apparent in the *Hakea* and *Eucalyptus* congeneric comparisons (Table 1). Our sampling strategies tried to maximize the variation in LMA between species that typify the sclerophyllous evergreen leaves of vegetation in temperate Australia. If LMA is causally related to characters that affect PNUE, then the relationship should be evident in the 10-fold range in LMA between our samples. We focused on measuring nitrogen allocated to leaf structure to explain variation in PNUE because few previous studies have done so. A possible reason for this has been the lack of simple and robust methods for separating cell walls and their bound protein from the remainder of the cell. The trade-off between nitrogen allocated to Rubisco and cell walls seen in *Quercus* (Takashima *et al.* 2004) and *Polygonum* (Onoda *et al.* 2004) was not confirmed with the Australian species examined here.

Relationship between the fraction of leaf nitrogen in cell walls and LMA

The fraction of leaf nitrogen in cell walls can be factored into three components: (1) the fraction of leaf biomass in cell walls; (2) the nitrogen concentration of cell walls; and (3) the nitrogen concentration of the whole leaf. While the nitrogen concentration of leaf material has been routinely measured, there are few data available for components 1 and 2. The fraction of leaf biomass in walls ranged from 0.20 to 0.65, and varied between genera. A considerably greater fraction of leaf biomass was recovered in the cell wall material from *Banksia* and *Hakea* leaves compared to *Acacia* and *Eucalyptus* leaves with similar LMA (Fig. 1). The fraction of leaf biomass in cell walls was independent of changes in LMA for the nine *Eucalyptus* species and all other species pairs except *Hardenbergia*.

Plant cell walls contain between 2 and 10% protein (Brett & Waldron 1996), which equates to 0.2–1.1 mmol N (g cell wall)$^{-1}$. Our values were consistent with this, ranging from 0.1 to 0.9 mmol N (g cell wall)$^{-1}$. Cell wall nitrogen concentration was about 0.4 times leaf nitrogen concentration for all genera apart from *Eucalyptus* where the factor was 0.6 (Fig. 2). Consequently, variation in cell wall nitrogen concentration was cancelled out by equivalent changes in leaf nitrogen concentration (see Eqn 2).

We carried out extensive comparisons between methods for extracting leaf material. Our initial extractions were done without PVP as we found that it adhered to some samples, and because it contains 12% N, minor contamination easily distorted the value derived for cell walls in these leaves with low nitrogen concentrations. The inclusion of PVP required multiple washing steps, including the use of 0.2 M KOH, to completely remove the 'soluble' PVP from the cell wall material. The difference between including PVP or not was most evident with the *Eucalyptus* species, and the data obtained without PVP are available on request.

In contrast to the strong positive relationship between the fraction of leaf nitrogen in cell walls and LMA in four *Quercus* species (Takashima *et al.* 2004) and *Polygonum* (Onoda *et al.* 2004), these parameters were independent in our study (Fig. 3). We conducted additional measurements with leaves collected from mature trees of two oak species to test the cell wall extraction methods. The evergreen *Quercus suber* had greater LMA than the deciduous *Quercus acutissima*, but a slightly smaller fraction of nitrogen in cell walls (0.106 ± 0.004 and 0.113 ± 0.012, with LMA values of 140 and 116 g m^{-2}, respectively). By contrast, the evergreen *Quercus acuta* and *Quercus glauca* had much smaller LMA values of 40–60 g m^{-2}, but similar cell wall nitrogen fractions between 0.1 and 0.2 (Takashima *et al.* 2004). The fraction of nitrogen in cell walls is independent of LMA when one considers all of the *Quercus* data together. About 0.06 of leaf nitrogen was recovered in cell walls from both sun and shade leaves of *Lindera umbellata*, although they differed 2.5-fold in LMA (Yasumura, Hikosaka & Hirose 2006). Therefore, it is not valid to generalize from the results of Onoda *et al.* (2004) and Takashima *et al.* (2004). When more species are included, one sees that the fraction of leaf nitrogen in cell walls is independent of LMA.

Variation in PNUE reflects changes in nitrogen allocated to Rubisco

The majority of variation in light-saturated PNUE between species can be attributed to factors affecting the supply of CO_2 to the sites of carboxylation (e.g. stomatal conductance, internal conductance) or the Rubisco activity per unit leaf nitrogen (because of nitrogen allocation, kinetic properties and activation state of Rubisco) (Pons, van der Werf & Lambers 1994; Hikosaka *et al.* 1998; Poorter & Evans 1998; Ripullone *et al.* 2003; Warren & Adams 2004). To facilitate the analysis of the underlying causes, we normalized the rates of CO_2 assimilation to a common intercellular CO_2 mole fraction of 300 μmol mol^{-1} (A_{a300}). Some of the remaining variation in PNUE could still be associated with variation in internal conductance, which influences the mole fraction of CO_2 in the chloroplast, C_c. None of the fourfold variation in PNUE observed by Lloyd *et al.* (1992) between four tree species was related to variation in C_i–C_c. Two reviews comparing sclerophyllous and mesophytic leaves also showed no difference in C_i–C_c if the comparison was restricted to the range where the photosynthetic rates overlapped (Evans 1999; Warren 2008). While there is a tendency for leaves with greater LMA to have lower internal conductances (Flexas *et al.* 2008), as they also tend to have lower photosynthetic capacities, the draw down C_i–C_c is not significantly related to LMA. A comparison between seven *Banksia* species ranging in LMA from 130 to 480 g m^{-2} also found that the draw down C_i–C_c was independent of LMA (Hassiotou, personal communication). Although it was not feasible to measure internal conductance in the present study, the values we observed for the carbon isotopic composition, Δ, and the extensive literature reviews suggest that it is unlikely that internal conductance accounts for the observed variation in PNUE.

Instead, nitrogen allocation to Rubisco, its kinetic properties and activation state are the probable causes for variation in PNUE. While there are several methods used to measure Rubisco content of leaves, it is not always possible to reliably extract Rubisco from some species. Consequently, an alternative approach is to derive Rubisco activity from gas exchange data using the Farquhar *et al.* (1980) model of photosynthesis. This can be converted to an amount of protein or nitrogen by assuming a catalytic turnover number, k_{cat}. Assuming constant values for Rubisco, kinetic parameters provide a convenient way of approximating Rubisco content, but conceal the variation which undoubtedly exists between species (k_{cat} values range between 2 and 6 mol CO_2 (mol Rubisco sites)$^{-1}$ s^{-1} (Seemann, Tepperman & Berry 1981; Seemann & Berry 1982; Makino, Mae & Ohira 1988; Evans 1989; Poorter & Evans 1998; Sage 2002; Ghannoum *et al.* 2005). There are several examples demonstrating good agreement between direct quantification of Rubisco and that calculated from gas exchange, such as for *Phaseolus vulgaris* (von Caemmerer & Farquhar 1981), *Triticum aestivum* (Evans & Seemann 1984), *Nicotiana tabacum* (von Caemmerer *et al.* 1994) or *Pseudotsuga* predicted using Rubisco kinetic parameters from *Nicotiana* (Ethier *et al.* 2006). However, Rubisco derived from gas exchange should be regarded as a minimum nitrogen cost because it is usually calculated assuming that all the Rubisco is fully activated, which was not the case for older *Pseudotsuga* leaves (Ethier *et al.* 2006). Assembling published data for different species reveals that the fraction of leaf nitrogen allocated to Rubisco declines curvilinearly as LMA increases (Fig. 6).

Trade-off between the fraction of leaf nitrogen in cell walls and Rubisco

The fraction of leaf nitrogen in cell walls was independent of LMA when examined across species and showed no consistent pattern within genera (Fig. 3). Therefore, it cannot be contributing to the negative relationship between PNUE and LMA. However, it is appealing to think that the allocation of nitrogen between different pools within a leaf involves competitive trade-offs and that variation in the allocation of nitrogen to cell walls could impact on nitrogen allocated to photosynthesis. The majority of nitrogen in a leaf is directly associated with photosynthesis (Evans & Seemann 1989; Makino & Osmond 1991; Pons *et al.* 1994; Hikosaka & Terashima 1995; Evans 1996; Niinemets & Tenhunen 1997; Poorter & Evans 1998). There are two major pools, the proteins of the chloroplast thylakoid membranes involved in light capture, electron transport and photophosphorylation, and the soluble proteins of the Calvin and photorespiratory cycles. To a first approximation, the sum of thylakoid and soluble protein gives a guide to the nitrogen cost of photosynthesis for a leaf. This cost was quite similar across 10 species representing herbaceous and woody plants, being four times the nitrogen content of Rubisco (Evans & Poorter 2001). However, for pea leaves, the sum of chloroplast plus mitochondrial nitrogen was three times the nitrogen content of Rubisco (Makino & Osmond 1991) and for *Eucalyptus cladocalyx*, total protein nitrogen was three times the nitrogen content of Rubisco (Gleadow, Foley & Woodrow 1998). This cost (three to four times Rubisco N) allows one to scale the nitrogen trade-off between photosynthesis versus leaf structure. The shaded zone in Fig. 7 represents the upper bound of this trade-off. For points falling within the shaded zone, all of leaf nitrogen would be accounted for by photosynthesis and cell wall material, leaving none for other cellular functions. Our data fall below this zone, suggesting that little soluble protein inadvertently stuck to the cell wall pellet, leaving 5–10% of leaf nitrogen unaccounted for.

Increased allocation of nitrogen to structure was accompanied by a reduced investment in Rubisco for both *Polygonum* (Onoda *et al.* 2004) and *Quercus* (Takashima *et al.* 2004) leaves. However, for *Polygonum*, the slope of the relationship (–1.5) greatly exceeded a direct trade-off (–0.25 to –0.33). Compared to deciduous *Quercus*, evergreen *Quercus* leaves increased nitrogen allocated to cell walls and decreased nitrogen allocated to Rubisco (Takashima *et al.* 2004), but the slope (–0.56) again exceeded a direct trade-off. A third study sampled leaves of *Lindera umbellata* throughout a growing season (Yasumura *et al.* 2006). This revealed that allocation of nitrogen to cell walls increased early in the season without any concomitant change to Rubisco and then during autumn, nitrogen released from Rubisco degradation was also not associated with any change in cell wall nitrogen. Clearly, there was no internal trade-off in nitrogen allocation between cell wall and Rubisco through the lifespan of these leaves. The spread of the *Eucalyptus* data revealed no correlation between Rubisco and cell wall nitrogen (Fig. 7), and the *Acacia* species pair from the ANBG actually had greater nitrogen allocation to Rubisco as the fraction of nitrogen allocated to cell walls increased. Considering all of the data together reveals that the 23 species populate a large part of the space below the shaded zone. The striking trade-off between nitrogen allocated to Rubisco versus cell walls observed for deciduous and evergreen *Quercus* is therefore unlikely to hold as a general rule. Although we were only able to assay cell wall nitrogen and had to calculate Rubisco nitrogen from gas exchange, our results refute the generality of the claim that increasing amounts of cell wall nitrogen in leaves with greater LMA necessarily result in a reduction in nitrogen allocation to Rubisco.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Leaf morphological, gas exchange and biochemical mean values for the congeneric species sampled in the Australian National Botanic Gardens and for the *Eucalyptus* species grown in the greenhouse.

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