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LETTER

C_4 photosynthesis boosts growth via altered physiology, allocation and size

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C_4 photosynthesis is a complex set of leaf anatomical and biochemical adaptations that evolved more than 60 times to boost carbon uptake compared to the ancestral C_3 photosynthetic type[1-3]. Although C_4 photosynthesis has the potential to drive faster growth rates[4,5], experiments directly comparing C_3 and C_4 plants have not shown consistent effects[1,6,7]. This is problematic, because differential growth is a crucial element of ecological theory[8,9] explaining C_4 savanna responses to global change[10,11], and research to increase C_3 crop productivity by introducing C_4 photosynthesis[12]. Here, we resolve this long-standing issue by comparing growth across 382 grass species, accounting for ecological diversity and evolutionary history. C_4 photosynthesis causes a 19-88% daily growth enhancement. Unexpectedly, during the critical seedling establishment stage, this enhancement is driven largely by a high ratio of leaf area to mass, rather than fast growth per unit leaf area. C_4 leaves have less dense tissues, allowing more leaves to be produced for the same carbon cost. Consequently, C_4 plants invest more in roots than C_3 species. Our data demonstrate a general suite of functional trait divergences between C_3 and C_4 species, which simultaneously drive faster growth and greater investment in water and nutrient acquisition, with important ecological and agronomic implications.

The repeated emergence of C_4 photosynthesis across multiple independent plant lineages transformed plant evolutionary history, and represents a remarkable example of convergent evolution[1,2]. Despite accounting for only 3% of extant plant species, C_4 lineages today dominate warm, open environments and account for 25% of terrestrial carbon fixation[9,10]. C_4 grasses include some of the world's most important food and energy crops, and C_4 grassy savannas provide critical ecosystem services for more than a

billion people[13]. Understanding how the C_4 photosynthetic pathway changes plant growth is therefore crucially important for plant evolution, crop production and ecosystem ecology.

Models of crop production and ecosystem dynamics assume that C_4 species have higher rates of photosynthesis than C_3 species under hot and sunny conditions, which lead to faster growth[5,10]. However, the numerous direct comparisons of C_3 and C_4 plant production made over the last 50 years have not consistently shown a growth rate advantage associated with C_4 photosynthesis (reviewed elsewhere[1,6,7]). We hypothesize that this inconsistency arises from the large variation in growth rates among ecologically diverse species, coupled with low statistical power arising from small sample sizes. Others have argued that it may arise from environmental limitations, or differences in internal resource consumption and allocation between C_3 and C_4 species[6,7].

To address this long-standing biological problem, we used an exceptionally large screening experiment to compare 382 grass species grown under controlled environmental conditions, within a phylogenetic and ecological framework. Species were sampled broadly across the two main clades of the Poaceae (grasses): i) the BEP lineage, comprised solely of C_3 species; and ii) the PACMAD lineage, which includes 22-24 independently evolved C_4 lineages[14], and a smaller but still substantial number of C_3 species. The sampling was structured to encompass species from different climate regions, characterised by alternative temperature (tropical and temperate), precipitation (arid and wet/humid) and tree cover (forested and open landscapes) combinations (Fig. 1). The species sample also incorporated both annual and perennial plants, and wild and domesticated crop species (Supplementary Table 1), but excluded bamboos, from which seeds are difficult to source. Ten plants per species were grown for five weeks under resource-rich, tropical conditions. Destructive harvesting over this period allowed us to

estimate species-specific relative growth rate (RGR) using a non-linear growth model. Accounting for size in this analysis is critical because RGR often declines as plants become larger[15]. RGR values were therefore estimated from growth models at small (20^{th} percentile of the biomass distribution across harvests for all species) and large (60^{th} percentile) sizes. The sizes were chosen as extremes in the size range over which all species were destructively sampled (excluding small or large sizes which were not attained by all species).

We resolved the key drivers of variation in RGR using a phylogenetic comparative analysis, selecting explanatory variables on the basis of *a priori* expectations about the ecological and life history traits that influence growth rate[16]. Life history (annual/perennial) had a large and highly significant effect, with annuals growing faster than perennials (Fig. 1; Supplementary Table 2). The other factors made smaller contributions, suggesting unexpectedly that neither adaptations to climatic region (tropical/temperate, humid/arid) nor specialisation within extreme habitats (waterlogged or dry soils, and open or shaded forest environments) exert strong effects on growth. Once all of these factors were taken into account, and evolutionary history was considered, the evolution of C_4 photosynthesis across the 16 origins sampled in our experiment had a major positive impact on growth (Fig. 1; Supplementary Table 2).

The positive effect of C_4 photosynthesis on growth under controlled tropical conditions was detected at both small and large plant sizes, but its magnitude was highly size-dependent (Fig. 2). The acquisition of C_4 photosynthesis generated a 19%, or 0.039 g g^{-1} d^{-1} increase in RGR at a small plant size (df=1,350, t=6.26, Pagel's λ=0.48, $p<0.001$; Supplementary Table 2, see also Fig. 2). However, at a large plant size, this effect increased to 88%, or a 0.125 g g^{-1} d^{-1} difference in RGR between the C_3 and C_4 species (df=1,330, t=5.27, Pagel's λ=0.29, $p<0.001$; Supplementary Table 2, see also Fig.

2). This strong size-dependence arose because the maximum plant size was five-times larger in C_4 than C_3 species (0.70g *vs* 0.12g, respectively: df=1,380, t=5.23, Pagel's λ=0.54, $p<0.001$), allowing C_4 species to continue growing faster for longer (Supplementary Fig. 1). The inclusion of bamboos would be unlikely to influence this result because these belong to the BEP clade, and are an outgroup to the direct comparisons between closely related C_4 and C_3 PACMAD species.

Growth rate variation can be decomposed into three components, representing: 1) the leaf area relative to leaf mass (specific leaf area, SLA); 2) the mass allocated to leaves relative to total plant mass (leaf mass ratio, LMR); and 3) the growth rate per unit leaf area (net assimilation rate, NAR). Almost since the discovery of C_4 photosynthesis, it has been assumed that higher photosynthetic rates increase the growth of C_4 species per unit of leaf area[17], i.e. through an increased NAR. Previous pairwise comparisons of C_3 and C_4 species have shown this expected effect (e.g. ref. 18). However, in large, multi-species comparisons, there has been little evidence either that high area-based photosynthesis generally translates into greater NAR[19] or that NAR is a major component of growth in C_4 species[20]. In our experiment, we also found no significant difference in NAR between C_4 and C_3 species at small sizes (df=1,380, t=0.95, Pagel's λ=0.50, p=0.34; Fig. 3). However, the difference in NAR between C_4 and C_3 species increased dramatically at large plant sizes, with values of 1.87 and 0.96 mg m^{-2} d^{-1}, respectively, resulting in a near-doubling in C_4 compared to C_3 species (df=1,341, t=4.80, Pagel's λ=0.36, $p<0.001$). The difference in NAR underpinned 82% of the variation in RGR at large plant sizes (Fig. 3; Supplementary Table 3), and is consistent with faster net carbon assimilation in the C_4 species. Photosynthesis in the C_4 species could have been further enhanced in the high light conditions of our experiment by greater canopy

temperatures and improved shoot water relations arising from lower stomatal conductance in C_4 than C_3 species[20,21].

Whilst NAR was not important for growth in small plants, SLA (specific leaf area) had a substantial effect. Values of SLA were 33% greater in C_4 than C_3 species at small plant sizes, 436 cm^2 g^{-1} compared to 328 cm^2 g^{-1}, respectively (*df*=1,380, *t*=3.5, λ=0.54, $p<0.001$). Variation in SLA was the main driver of variation in RGR (Fig. 3; Supplementary Table 3). At large sizes, SLA was still 39% higher in C_4 than C_3 species (*df*=1,380, *t*=4.28, λ=0.49, $p<0.001$) (Fig. 3; Supplementary Table 3). The high productivity of C_4 species is therefore driven by higher SLA at small plant sizes, and a longer period of rapid growth during which NAR becomes increasingly important (Supplementary Figs. 2-3 and Supplementary Discussion).

For plants in general, the maintenance of a large root system carries a respiratory burden, which reduces the availability of carbon for growth[19]. Fast growth is therefore often achieved via greater allocation to shoots relative to roots[19], but this may compromise root properties that depend on size, such as competition for below-ground resources[22] and the belowground storage of energy reserves[23]. Our data provide strong empirical support for the hypothesis[7] that C_4 photosynthesis allows plants to fundamentally change this inherent trade-off between growth and allocation. Despite growing faster than C_3 species, C_4 plants allocated 54% more biomass to roots, with root mass ratios (root mass/plant mass) averaging 0.46 in C_4 and 0.29 in C_3 plants (*df*=1,380, *t*=5.54, λ=0.40, $p<0.001$; Supplementary Fig. 4). This simultaneous adjustment of growth and allocation in C_4 species permits a diverse range of novel ecological strategies and opportunities that are unavailable to C_3 plants[7].

It has been noted previously that C_4 species may have high SLA (or a low investment of leaf mass per unit area)[24]. However, the generality of this observation and

its full significance for the high productivity of C_4 species has not been appreciated. We also sought a mechanistic explanation for the SLA variation among species in terms of leaf thickness and density, following previous authors in using leaf dry matter content (LDMC, the ratio of leaf dry mass to fresh mass) as a proxy for density[25]. Leaf thickness did not differ significantly between C_3 and C_4 species (df=1,380, t=0.34, λ=0.51, p=0.74). However, variation in LDMC accounted for 70% of the variation in SLA between C_3 and C_4 species. The LDMC was significantly lower in C_4 than C_3 species, with a value of 18% compared to 23%, respectively (df=1,380, t=3.50, λ=0.52, p<0.001). Approximately 25% of the between-species variance in leaf density was linked to changes in photosynthetic pathway (Supplementary Table 4). At first sight, this result seems puzzling, since proportions of high-density tissues such as bundle sheath and sclerenchyma are higher in C_4 than C_3 leaves[26], and proportions of air spaces are lower[27]. Instead, the overall difference in leaf density must arise from other changes in tissue density associated with the C_4 pathway, including: a decrease in the proportion of cell walls caused by larger cells or thinner cell walls; a decrease in the total protein content driven by reduced mesophyll investment in C_3 cycle enzymes, which include the most abundant proteins in plants[7,28]; or a difference in vascular structure and architecture, associated with leaf hydraulics[29].

Controlled environment screening experiments of the kind performed here can provide important insights into the fundamental differences in growth characteristics among species. However, they necessarily focus on plants during the early phase of rapid growth, so that many species remain immature for the duration of the experiment. Furthermore, the application of a simplified, common environment is inevitably sub-optimal for the growth of certain species. The general significance of the growth characteristics identified here must therefore be tested for mature plants under field

conditions, where responses are mediated by abiotic (e.g. drought limitations) and biotic (e.g. mycorrhizal symbioses) interactions. Similarly, the ecological adaptations to climatic region and extreme habitats considered here are necessarily coarse global descriptors of ecology, and do not capture finer scale ecological adaptations to factors like soil fertility, which are known to influence RGR[16].

We have clarified an important and long-standing controversy in the literature via a phenotyping study of unprecedented scale, coupled to an analysis that accounted for ecology and evolutionary history simultaneously. These novel aspects of the work have enabled us to demonstrate profound effects of C_4 photosynthesis on growth and allocation, a strong size-dependence in these effects that is linked to maximum plant size, and a central mediating role for leaf construction costs. The effects were resolved for juvenile plants within a controlled environment and might be altered in mature plants in natural environments by local environmental processes acting at the population or species levels. The work therefore highlights the vital importance of using comparative screening in controlled environments to study physiological innovations within the context of the whole organism. It has crucial implications for the ecological behaviour and interactions of species in grassy biomes, and for the introduction of novel physiological traits into crops to improve yields.

[Main Text, including introductory paragraph 2,042 words]

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature, and includes:

Supplementary Methods

Supplementary Discussion

Supplementary Figures 1-5

Supplementary Tables 1-5

References

Supplementary Data 1: phylogenetic trees

Trait data: a complete species-level dataset has been deposited at Dryad doi <*fill in details if paper is accepted*>

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Figure Legends

Figure 1. Relative growth rate of the sampled species according to photosynthetic pathway, life history, realised climatic niche and phylogeny.

From the centre, the black tips of the phylogeny indicate annual species, and the grey tips perennials. The inner red-coloured circle represents mean annual temperature across the distribution of each species, while the outer blue-coloured circle represents mean annual precipitation. The length of bars around the outside are scaled to represent relative growth rate (RGR) in small plants (at the 20^{th} size percentile), with red bars showing C_4 species and black bars C_3 species in the PACMAD and BEP clades of Poaceae (grasses).

Figure 2: Relative growth rate.

a. Relative growth rate (RGR) in small (20^{th} size percentile) and **b.** large (60^{th} size percentile) plants. Comparisons between sister C_3 (black) and C_4 (red) lineages are highlighted by coloured shading and linked points. For each lineage, means and standard errors were calculated from raw data. Error bars are not visible in some cases because they are smaller than the symbol, whereas in other cases lineages are represented by a single species (Supplementary Table 1 for the species in each lineage). The overall difference between C_3 and C_4 species calculated in a phylogenetic analysis is shown at the bottom of each panel.

Figure 3. Components of growth.

Net assimilation rate (NAR, **a**, **d**), specific leaf area (SLA, **b**, **e**) and leaf mass ratio (LMR, **c**, **f**) in small (20^{th} size percentile, **a, b**, **c**) and large (60^{th} size percentile, **d e**, **f**)

plants. Comparisons between sister C_3 (black) and C_4 (red) lineages are highlighted by coloured shading and linked bars. For each lineage, means and standard errors were calculated from raw data, and the overall difference between C_3 and C_4 species is at the bottom of each panel. Error bars are not shown in some cases for the reasons outlined in Fig. 2.

METHODS

Growth experiment. Seeds were obtained from seed banks, commercial suppliers, or the wild, and sterilized prior to germination (Supplementary Table 1). Seedlings were transplanted into 1-litre pots (Length: 5cm, Width: 5cm, Height: 40cm), containing 90% vermiculite and 10% sand by volume.

The experiment compared the growth of 382 species under the same environmental conditions. We used a controlled environment chamber (MTPS 120, Conviron, Winnipeg, Manitoba, Canada) to provide a day/night temperature of 30°C/25°C and 70% RH. Daylength in the chamber was 14 h, with a maximum photosynthetic photon flux density measured at canopy height of 1,600 μmol m^{-2} s^{-1}. We aimed for a non-limiting water supply by watering twice daily using an automated irrigation system, and a non-limiting nutrient supply by feeding twice a week with 50% nitrate-type Long Ashton solution[30].

Individual plants were harvested approximately weekly for five weeks. Harvested plants were washed, and fresh weight and total leaf area determined (WinDIAS Image Analysis System, Delta-T Devices, Cambridge, UK). Plant material was dried at 70 °C to a constant mass, and leaf, stem (including leaf sheath and culm), and root fractions weighed independently.

Full details of the experiment are provided in Supplementary Methods.

Growth analysis. All statistical analyses used the R language and environment (R Foundation for Statistical Computing, Vienna, Austria, 2013). Total plant dry mass over time was used to model species-specific growth curves. For all traits, we calculated both an average value per species, and a predicted value at two common sizes; the 20% and 60% percentiles for total plant dry weight across all species and all harvests. Each trait at

the 20% and 60% percentiles was estimated using linear regression against total plant dry weight.

To ensure that our estimates of growth rate were robust, we fitted a wide range of growth models. In all cases, we modelled ln(mass) as a function of time, and included terms for experiment and block as fixed effects. Details of the models, fitting methods, derivation of growth rates, and comparisons between the models, are provided in the Supplementary Methods. RGR values are reported from the 4-parameter logistic model.

Components of growth. RGR can be broken down into three components, NAR, SLA and LMR. We looked at the relationships between the components, RGR and the C_4 pathway at several different levels: 1) whether the growth component values differ significantly between photosynthetic types; 2) how much each component contributes to the variance in RGR, (see ref. 15); and 3) whether the variance in the growth components is due to the C_4 pathway or species-specific differences, using a new variance decomposition method (see Supplementary Methods).

Specific leaf area (SLA) can be further decomposed as:

$$SLA = \frac{leaf\ area}{leaf\ mass} = \frac{1}{leaf\ thickness\ x\ leaf\ density}.$$

However, accurate direct measurements of leaf density and thickness are difficult under the constraints of a large experiment. Leaf dry matter content, was therefore determined as an easily obtained proxy for leaf density[25]. With this information, estimates of leaf thickness can be derived by assuming invariance in the density of fresh leaves among species[25].

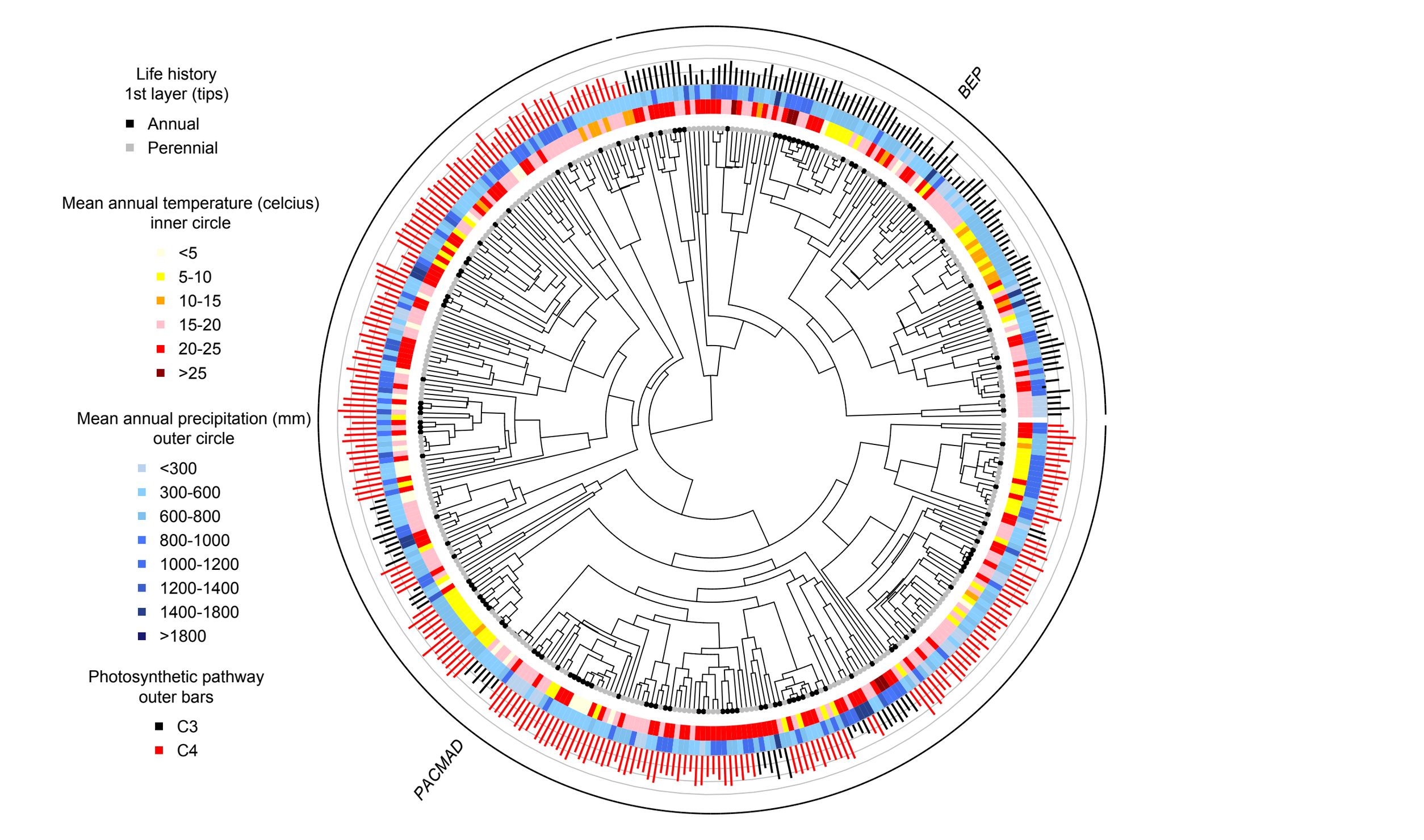
Habitat characterisation. Published information about the habitats occupied by each species was collected and categorized into wetness and shadiness categories designed to distinguish specialists of wetlands, shallow and free-draining soils, or forest shade environments from other species. We also collated published information about annual / perennial life history, maximum plant height in the field, whether the species was sod-forming (had rhizomes and/or stolons) or not, and its domestication status. Broad climatic zones (i.e. tropical vs temperate, arid vs wet/humid) were distinguished using mean annual precipitation (MAP) and mean annual temperature (MAT) across the geographical range of each species. Full details are provided in the Supplementary Methods.

Comparative analysis. A phylogeny was reconstructed using new sequences combined with data retrieved from the NCBI database (see Supplementary Methods and Supplementary Data 1). For the comparative analysis, we used gls in the nlme package, applying the maximum likelihood method to calculate the phylogenetic signal, Pagel's lambda (λ). We fitted the explanatory variables based on *a priori* expectations of which factors might influence plant species growth. In this analysis we accounted for life history (annual or perennial), domestication status (wild or cultivar), MAT and MAP across the species distribution, habitat wetness (xeric, dry, wet, very wet, waterlogged), habitat shadiness, (open, closed, broad), plant height in the field, and growth form (sod-forming or not sod-forming). Comparative analyses across all growth models ensured that the key results were robust to statistical approach (see Supplementary Table 2).

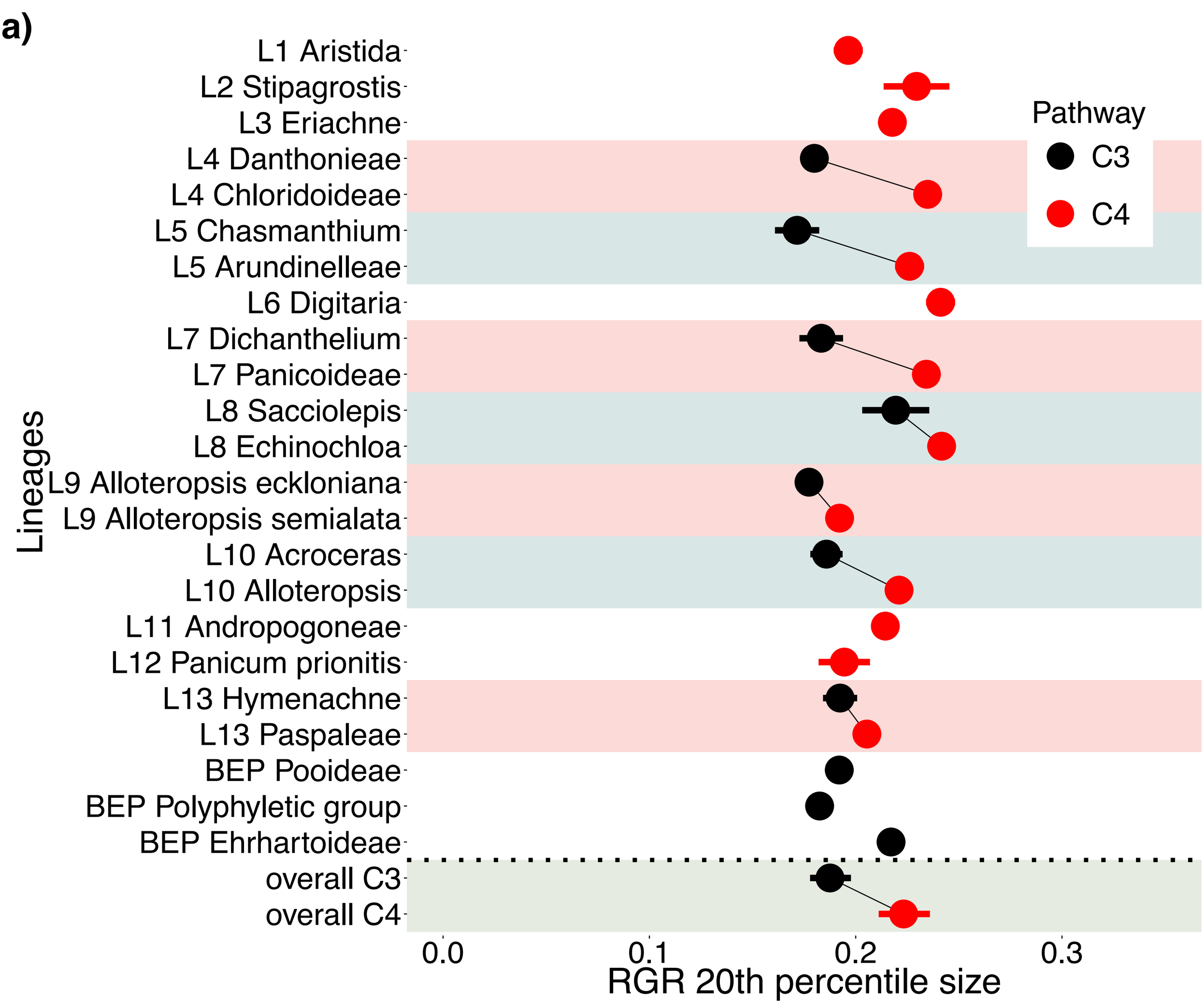
We did not reduce or simplify the models, principally because all variables were expected to have a significant influence on growth. We present our main comparative model without interactions, but did test two-way interactions individually for all

variables, and used a 95% confidence test in the ANOVA table to reject or accept interactions. We then looked at whether the interaction was biologically meaningful.

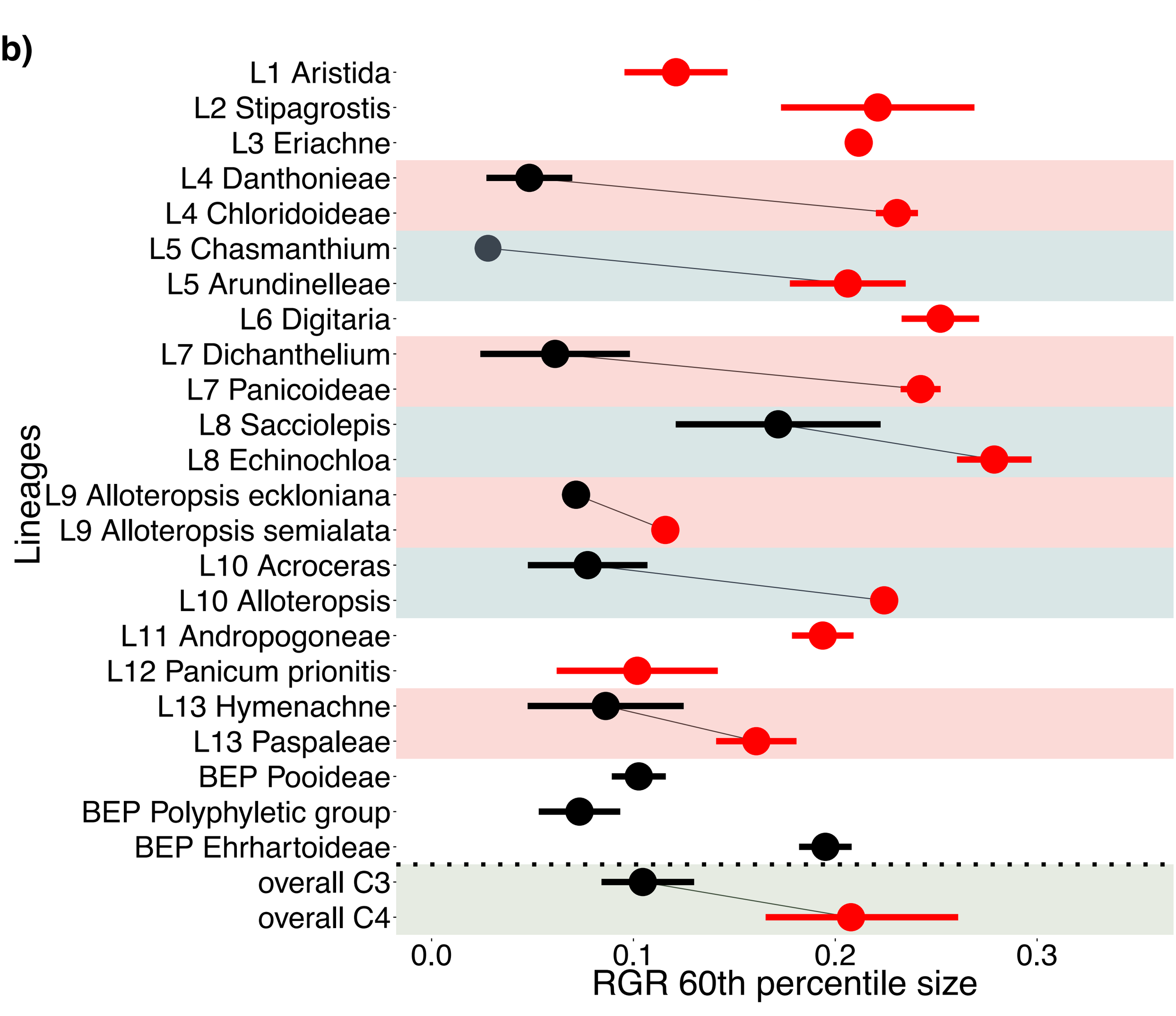
[Methods 805 words]

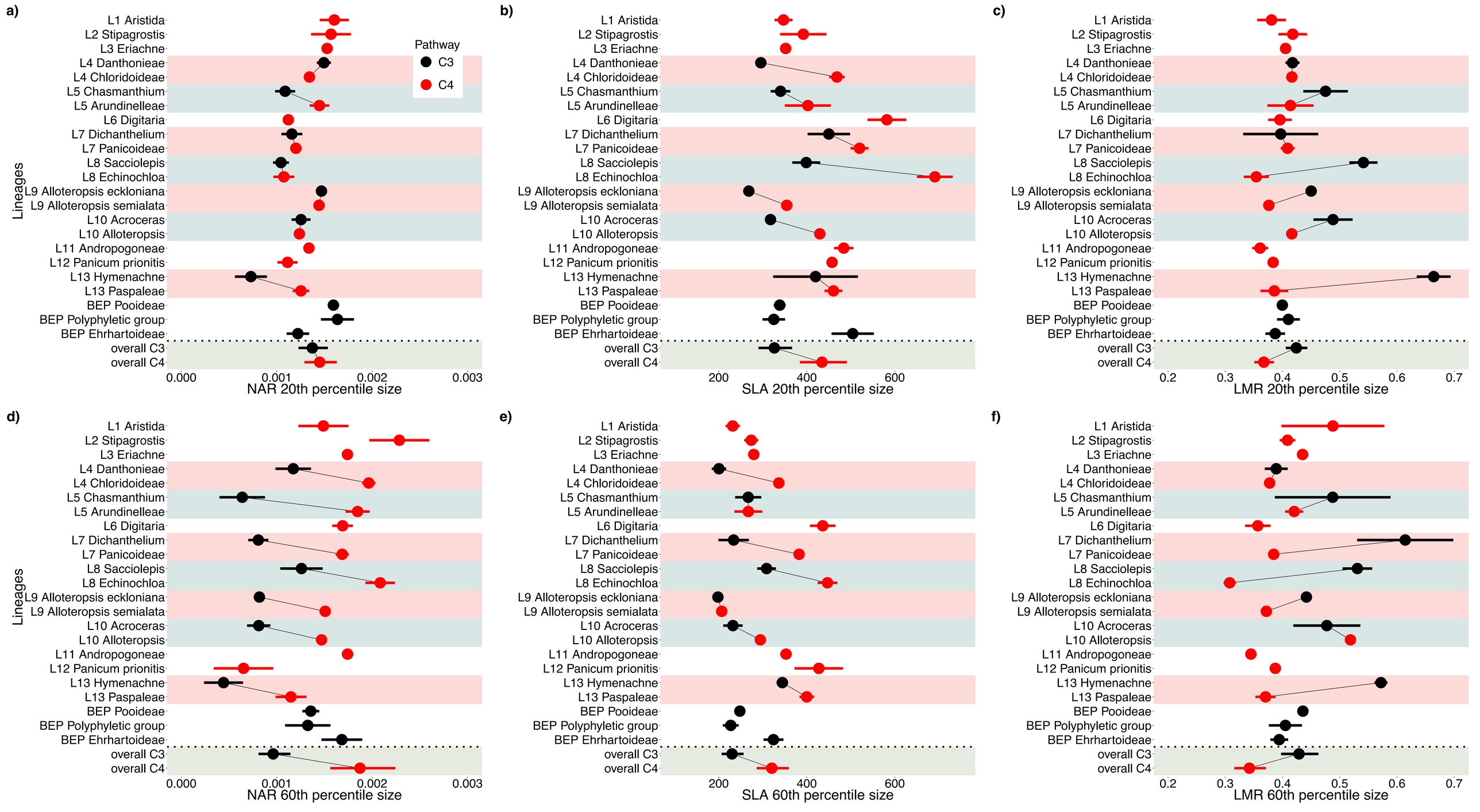

Life history
1st layer (tips)
Annual
Perennial
Mean annual temperature (celcius)
inner circle
<5
5-10
10-15
15-20
20-25
>25
Mean annual precipitation (mm)
outer circle
<300
300-600
600-800
800-1000
1000-1200
1200-1400
1400-1800
>1800
Photosynthetic pathway
outer bars
C3
C4
BEP
PACMAD

a)


Lineages
L1 Aristida
L2 Stipagrostis
L3 Eriachne
L4 Danthonieae
L4 Chloridoideae
L5 Chasmanthium
L5 Arundinelleae
L6 Digitaria
L7 Dichanthelium
L7 Panicoideae
L8 Sacciolepis
L8 Echinochloa
L9 Alloteropsis eckloniana
L9 Alloteropsis semialata
L10 Acroceras
L10 Alloteropsis
L11 Andropogoneae
L12 Panicum prionitis
L13 Hymenachne
L13 Paspaleae
BEP Pooideae
BEP Polyphyletic group
BEP Ehrhartoideae
overall C3
overall C4
0.0
0.1
0.2
0.3
RGR 20th percentile size
Pathway
C3
C4

b)


Lineages
L1 Aristida
L2 Stipagrostis
L3 Eriachne
L4 Danthonieae
L4 Chloridoideae
L5 Chasmanthium
L5 Arundinelleae
L6 Digitaria
L7 Dichanthelium
L7 Panicoideae
L8 Sacciolepis
L8 Echinochloa
L9 Alloteropsis eckloniana
L9 Alloteropsis semialata
L10 Acroceras
L10 Alloteropsis
L11 Andropogoneae
L12 Panicum prionitis
L13 Hymenachne
L13 Paspaleae
BEP Pooideae
BEP Polyphyletic group
BEP Ehrhartoideae
overall C3
overall C4
0.0
0.1
0.2
0.3
RGR 60th percentile size


a)
b)
c)
d)
e)
f)
Lineages
L1 Aristida
L2 Stipagrostis
L3 Eriachne
L4 Danthonieae
L4 Chloridoideae
L5 Chasmanthium
L5 Arundinelleae
L6 Digitaria
L7 Dichanthelium
L7 Panicoideae
L8 Sacciolepis
L8 Echinochloa
L9 Alloteropsis eckloniana
L9 Alloteropsis semialata
L10 Acroceras
L10 Alloteropsis
L11 Andropogoneae
L12 Panicum prionitis
L13 Hymenachne
L13 Paspaleae
BEP Pooideae
BEP Polyphyletic group
BEP Ehrhartoideae
overall C3
overall C4
Pathway
C3
C4
0.000
0.001
0.002
0.003
NAR 20th percentile size
200
400
600
SLA 20th percentile size
0.2
0.3
0.4
0.5
0.6
0.7
LMR 20th percentile size
NAR 60th percentile size
SLA 60th percentile size
LMR 60th percentile size

Supplementary Information

Supplementary Methods

Seed sterilization and germination

Seeds were supplied by seed banks (Kew Millennium Seed Bank, USDA, IPK, CIAT and the Australian Plant Genetic Resource Information Service), commercial seed suppliers (B&T World Seeds, Herbiseed, Silverhill, Prairie Moon Nursery), or collected from the wild. Some species were bulked from original stock to ensure there was sufficient seed for experimental purposes.

The pericarps of all seeds were removed, and the seeds stored in a dry environment at 5^{o}C until the experiment. Prior to germination, twenty seeds of each species were mixed for three minutes in a saturated calcium hypochlorite solution to sterilize them. The seeds were then washed in distilled water over a Buchner funnel and plated onto filter paper wetted with distilled water in petri dishes.

Species-specific germination conditions were collated from the Seed Information Database (SID) of Kew Royal Botanic Gardens (http://data.kew.org/sid/germ.html), and the literature (64% of species). If species-specific germination conditions could not be found (36% of the species), C_3 species were germinated at 20^{o}C and C_4 species at 25^{o}C. This was to avoid germination failure due to a non-ideal temperature biasing the species sample.

Experimental design

Our study used a "common garden" experimental design, in which all species were grown and compared under the same environmental conditions. Because we wished to compare more species than we could fit into the growth chamber at one time, we split the experiment into two halves, run sequentially. There was no bias in the distribution of species between halves of the experiment. To statistically control for differences between these two halves of

the experiment, the analysis includes "replicate" as a factor in the analysis of the growth data. This statistical control was facilitated by including twenty species in both halves of the experiment.

The experiment was simply too large for it to be practical to rotate the plants regularly. Instead, we dealt with the inevitable heterogeneity of the growth environment using a randomized block design, and included a block effect in the statistical analysis. This design ensured that environmental heterogeneity did not introduce a systematic bias into the experiment. Upon germination, seedlings were therefore allocated a random location in the experimental chamber, and given a random harvest date 1, 2, 3, 4 or 5 weeks after transplantation. Coupling this approach with a curve-fitting method for estimating growth rates ensured that any effects of environmental heterogeneity (or, for example, the differential effects of transplant shock among individuals) on growth rate were minimized. The exact harvesting date was adjusted for some plants to maintain a similar range of sizes for RGR analysis (i.e. the harvest date for plants that were comparatively small at the designated harvest was often delayed, and brought forward for plants that were comparatively large). This approach maximised the overlap in sampled sizes among species.

Growth environment

We used a combination of large tungsten and metal halide lamps in the growth chamber for this experiment, beginning with new lamps to ensure maximum performance, and burning them in immediately before starting the experiment. Previous measurements have shown that output does drop off during the burn-in period but that, afterwards, lamp output is relatively stable on a timescale of months. After the lamp burn-in, we measured photosynthetic photon flux density (PPFD) at plant canopy height across the whole growth area using a handheld sensor (LI-COR 190-R quantum sensor, LI-COR Environmental, Lincoln, Nebraska, USA).

During the photoperiod of the experiment, the PPFD in the growth chamber was increased in three equal steps over a 4-hour period, held at a maximum measured value of 1,600 μmol m^{-2} s^{-1} for 6 h, and then stepped down again over another 4-hour period.

Heat load is managed in the growth cabinet by enclosing lamps within an air-conditioned compartment, with a Perspex window into the chamber. IR radiation emitted from the lamps is strongly absorbed by Perspex, and the Perspex itself is cooled using the air-conditioning to minimize sensible heat flux into the chamber. During the experiment, the majority of plant leaf canopies were 80-90cm below the Perspex window, allowing air to circulate freely.

Sand is normally used for growth analysis to enable easy root washing. However, several tons of wet sand would have been required for our experiment, which could not be supported by the growth cabinet shelves. We chose coarse vermiculite as an alternative growth medium because it is significantly less dense than sand, and pilot experiments had shown that it enabled both rapid root washing and a high recovery of fine roots. We found through this pilot experimentation that vermiculite flakes could be easily crushed between the fingers to liberate fine roots, and we washed all roots over a 2 μm mesh, so that the vast majority were recovered, even if they were broken during washing. A 2 cm layer of sand was laid over the vermiculite in the pots, as pilot experiments showed that this increased the likelihood of seedling establishment.

Growth analysis – models and fitting

To ensure that our estimates of growth rate were robust, we fitted a wide range of growth models, discarding species where we had biomass data for fewer than five individuals. In all cases, we modelled ln(mass) as a function of time, and included terms for experiment and

block as fixed effects. In the model description below we suppress the experiment and block effects to simplify presentation.

1) Linear growth model: $\ln(M) = a + bt$

We fitted this model in a phylogenetic framework using the `lmekin` function in the `coxme` package. The fitted model was

```
lmekin(logm ~ phylo.name + time + phylo.name:time +
(1|phylo.name) , data = data.f , varlist = list(vmat,
lambdamat))
```

This fits a linear model with fixed effects for the species-specific intercepts and slopes. The intercept is modelled as a random effect varying between species with two variance components. The first (`vmat`) is a phylogenetic variance-covariance matrix, while the second (`lambdamat`) is an identity matrix that captures variation between species unrelated to phylogeny. The estimated species-level random effects were both very small (variance < 5e-7) compared to the residual error (0.83) and so the model predictions were very similar to a standard linear model fitted with `lm` (r^2 for the model slopes 0.9997). It is not possible to fit random slope models in `lmekin`.

2) Quadratic growth models: $\ln(M) = a + bt + ct^2$

We fitted these models in a mixed model framework using the `lmer` function from the `lme4` package. These models do not allow the inclusion of phylogenetic effects, but random slope models can be fitted. We fitted a range of models and selected plausible models using AIC and BIC. The best model selected using BIC was

```
logm ~ time + I(time^2) + (1|phylo.name) + (1+time|phylo.name)
```

which has fixed effects for time and time-squared, and correlated, species-specific random effects for the intercept and time slope. AIC identified a more complex model of the form

```
logm ~ time + I(time^2) + (1|phylo.name) + (1+time|phylo.name)
+ (1+I(time^2)|phylo.name)
```

which is similar to the previous model but has a species-specific time-squared random effect that is correlated with the intercept. The predicted average RGR's (see below) were very similar (r^2=0.996) suggesting that the precise details of the fitted models were unimportant, and so we used the simpler model.

3) MCMC quadratic growth models: $\ln(M) = a + bt + ct^2$

We fitted these models using the `MCMCglmm` function from the `MCMCglmm` package, using 100,000 iterations and a thinning interval of 100. This allows very complex models to be fitted. The model we used was

```
logm ~ time  + I(time^2), random = ~idh(1 + time +
I(time^2)) : phylo.name + idh(1) : phylo.name.ide ,
ginverse=list(phylo.name=Ainv)
```

which has independent, phylogenetic random effects for the intercept, time and time-squared terms and an additional random effect describing variation between species independent of phylogeny in the intercept.

4) Four-parameter logistic: $\ln(M) = A + \frac{B-A}{1+\exp(-k(t-t_0))}$

We fitted these models using `nlme` from the `nlme` package. The asymptotic size, *B,* varied between experiments, while the parameters t_0 and *k* varied by block. All four parameters were fitted as independent, species-specific random effects.

Growth analysis – calculation of growth rates

For the linear model, where ln(mass) is regressed against time, we have a fitted model of the form

$$\ln(M) = a + bt$$

and so RGR, which is $\frac{dM}{Mdt}$, is simply *b*. For the quadratic model we have

$$\ln(M) = a + bt + ct^2$$

and so RGR is $b + 2ct$. As this is a function of time, we need to either select a time to compare species, say the end of the experiment, or calculate the average RGR over the course of the experiment. We used the latter approach. Average RGR is given by

$$\widetilde{RGR} = \frac{1}{T}\int_0^T RGR(t)dt = \frac{1}{T}\int_0^T \frac{dM}{Mdt}dt = \frac{1}{T}(\ln(M(T)) - \ln(\mathrm{M}(0))$$

where *T* is the duration of the experiment. Substituting the values for *M* from the quadratic model we find $\widetilde{RGR} = b + cT$. Note that, if we had used RGR at the end of the experiment, we would have used $RGR = b + 2cT$, which is very closely related to average RGR. For the four-parameter logistic model we have

$$\ln(M) = A + \frac{B - A}{1 + \exp\left(-k(t - t_0)\right)}$$

where A is the minimum mass, B the maximum, t_0 the time when a plant is midway between A and B, and k a growth parameter. For this model, the size-specific RGR is given by

$$\frac{k(A - \ln(M_c))(B - \ln(M_c))}{(A - B)}$$

where M_c is the mass at which RGR is calculated[1].

Growth analysis – comparison between models

We calculated RGR for the 4-parameter logistic model at both the 20th and 60th percentiles of the size distribution. These RGR estimates were compared with a classical linear regression model including the effects of phylogeny (model 1, above), a quadratic mixed effects model ignoring the effects of phylogeny (model 2, above), and a quadratic Bayesian mixed effects model including the effects of phylogeny (model 3, above). In all cases, regardless of how RGR was estimated, there was a strong positive relationship between the RGR measures (Supplementary Fig. 5; $p<0.0001$, $r^2>0.5$ in all cases).

Variation in the components of growth

We developed a method to partition the effect of C_4 photosynthesis on variation in the components of growth, as follows. First, the variance contribution to RGR from variation in *nar* is given by

$$Cont(nar) = \frac{Var(nar)+Cov(nar,sla)+Cov(nar,lmr)}{Var(rgr)} \quad 1$$

where *Var* is the variance and *Cov* the covariance[1]; note that lower case terms indicate logarithmic transformation (e.g. $nar = \log(NAR)$). We now take this variance partitioning further by separating the variance and covariance terms in equation 1 into variation due to species effects unrelated to photosynthetic pathway, and variation due to photosynthetic pathway. First we construct a linear model of the form:

$$nar = \beta_0 + \beta_{nar} I_{PP} + \varepsilon_{nar}$$

Where β_0 is the intercept, β_{nar} the effect of photosynthetic pathway on *nar*, I_{PP} an indicator variable with values 0=C_3, 1=C_4, and ϵ_{nar} is the error, which we will use as our estimate of how species vary independently of changes in *nar* related to photosynthetic pathway. Thus, the variance in *nar* is:

$$var(nar) = \beta_{nar}^2 Var(I_{PP}) + Var(\varepsilon_{nar})$$

We can interpret this as the variance as a result of variation in photosynthetic pathway ($\beta_{nar}^2 Var(I_{PP})$), plus the variation between species independent of photosynthetic pathway ($Var(\varepsilon_{nar})$). Note the $Cov(I_{PP}, \varepsilon_{nar}) = 0$ for least squares estimators. The covariance terms can also be partitioned in this way, so for $Cov(nar, sla)$ we have

$$Cov(nar, sla) = E\left[\left(\beta_{nar}(I_{PP} - \overline{I_{PP}}) + \varepsilon_{nar}\right)\left(\beta_{sla}(I_{PP} - \overline{I_{PP}}) + \varepsilon_{sla}\right)\right]$$

since $E[\varepsilon] = 0$. Expanding the terms within the square brackets, gives us four terms.

$$Cov(nar, sla) = \beta_{nar}\beta_{sla}Var(I_{PP}) + \beta_{nar}Cov(I_{PP}, \varepsilon_{sla}) +$$
$$\beta_{sla}Cov(I_{PP}, \varepsilon_{nar}) + Cov(\varepsilon_{nar}, \varepsilon_{sla})$$

The first three terms describe the variation linked to variation in photosynthetic pathway, whereas the fourth term describes the covariance between the species differences in *nar* and *sla* independent of changes in photosynthetic pathway. We can therefore partition the variance and covariance terms in equation 1 into components related to changes in photosynthetic pathway, and to species differences that are independent of the change in the trait linked to photosynthetic pathway. In order to assess which components of growth (SLA, NAR, LMR) were the most important for contribution to RGR, we looked at component values at a range of plant sizes, to account for differences that are due to allometric scaling, and present the 20% and 60% percentile plant sizes to represent small and large plants.

Habitat characterisation

Information about the habitats occupied by each species was collected from online and published floras and herbaria, and categorized. The habitat categories were shadiness (open, partly shaded, shaded), soil texture (clay, loam, silt, sand, rocky), pH (acidic, neutral, basic), altitude (0-500, 501-1000, 1001-1500, 1501-2000, 2001-2500, 2501-3000, 3001-3500, 3501-4000, 4001-4500, 4501-5000), salinity (glycophytic, salt-tolerant), and soil wetness (dry/arid, damp/moist/humid, wet/marshy, waterlogged/flooded).

For example, for the habitat description *"Forest margins and rocky mountain grasslands 1000–2700m. Clay or sand on shallow soil of forest margins or open grasslands, mainly in dark places and around boulders, grassland and forest"*, the species was

categorized as growing in ‘open’ or ‘part shade’ (shadiness category), ‘clay’ and ‘sandy’ soils (soil type category), at ‘1001-1500’, ‘1501-2000’ and ‘2001-2500m’ altitude (altitude category). For this example, there was no information for pH, salinity and moisture to include information in these categories. If there was no mention of a category in the habitat description, then NA was given for all category levels. However, if a category was described, then we assumed that the species did not occur in habitats that were not identified in the description.

We found that only the wetness and shadiness categories were populated with sufficient information across species and with enough variation among levels to include in the final comparative analyses. For these analyses, the different combinations of levels in habitat categories were further summarised (Supplementary Table 5).

We collated information about growth habit, (caespitose, rambling/creeping, matt-forming) from the literature and GrassBase[2]. However, there was insufficient variation in growth habit for the comparative analysis, with the vast majority of species being caespitose. We also collected data from the same sources on maximum culm height at flowering, and whether species were rhizomatous, stoloniferous or both. If information about rhizomes/stolons was absent, we assumed that the species had neither. The same sources also detailed whether the species was an annual or perennial, or whether it had been observed with both life histories. We simplified this information to annual or perennial, recoding to annual all instances where species could be either annual *or* perennial.

Where information about improvement status was provided by the seed distributor, this was summarised directly into ‘wild’ or ‘cultivar’ levels in the ‘domestication status’ category. The cultivar level included landraces and elite crops. Where seed improvement information was ‘uncertain’, the species was placed in the ‘wild’ category level.

For realised climatic niches, we used raster climate data files available at Worldclim[3] and species' occurrence information available at the Global Biodiversity Information Facility (GBIF) to extract information about species presence at the country level (present/absent) and current climate information for the country (mean annual temperature, MAT, and mean annual precipitation, MAP). We averaged values over countries where individual species occurred, weighted by country size, to obtain a single value for MAT and MAP for the species distribution. This was completed in the R statistical software[4], using an area-based re-projection of the global map.

Phylogenetic reconstruction

For all species included in this study, *PHLAWD*[5] was used to retrieve up to seven different markers: the nuclear ITS and chloroplast markers *trnKmatK*, *ndhF*, *psbA*, *rbcL*, *rpL16* and *trnLF*. Synonyms were identified using Grassbase[2], and in cases in which multiple sequences were available, only the longest sequence was retained. At least one of the seven markers was available for all but 52 of the included species. For these remaining 52 species, DNA was isolated from dried plant material using the DNeasy Plant Mini Kit (Qiagen Inc., Texas, USA), following the instructions provided. These gDNAs were used as PCR templates to amplify fragments of *trnKmatK*, *ndhF*, and ITS, following the protocols and using the primers previously publishe[6,7]. The newly generated sequences were added to those retrieved from NCBI database. Each marker was individually aligned using Muscle[8], and the alignment was manually refined. The seven datasets were then merged, producing one concatenated marker per species. The final dataset consisted of 444 species and 9674 aligned bp.

The final dataset was used to obtain a time-calibrated phylogenetic tree through Bayesian inference as implemented in BEAST[9]. The general time reversible substitution model with a gamma shape parameter and a proportion of invariants (GTR+G+I) was used. A

log-normal relaxed clock was used, with prior divergence times modelled by a Yule process. The tree was rooted by forcing the monophyly of each of the BEP and PACMAD sister clades (no species outside of these clades was included in the dataset). Their split was forced to follow a normal distribution, with a mean of 51.2 and a standard deviation of 6.0, following Christin *et al*.[10]. Three separate analyses were run for more than 37,000,000 generations, sampling a tree every 5,000 generations. The adequacy of the burn-in period (set to 20,000,000 generations) and convergence of the runs were verified using Tracer[11]. All the trees sampled post-burn-in were pooled, and medians of node ages were plotted on the maximum-credibility tree, which was used for comparative analyses. However, we repeated the analyses on a set of >100 of the posterior trees, and the results are almost identical (typically within a couple of decimal places) and the statistical significance of the experimental factors is unaltered in every case.

Supplementary Discussion

Contributions to growth rate variation

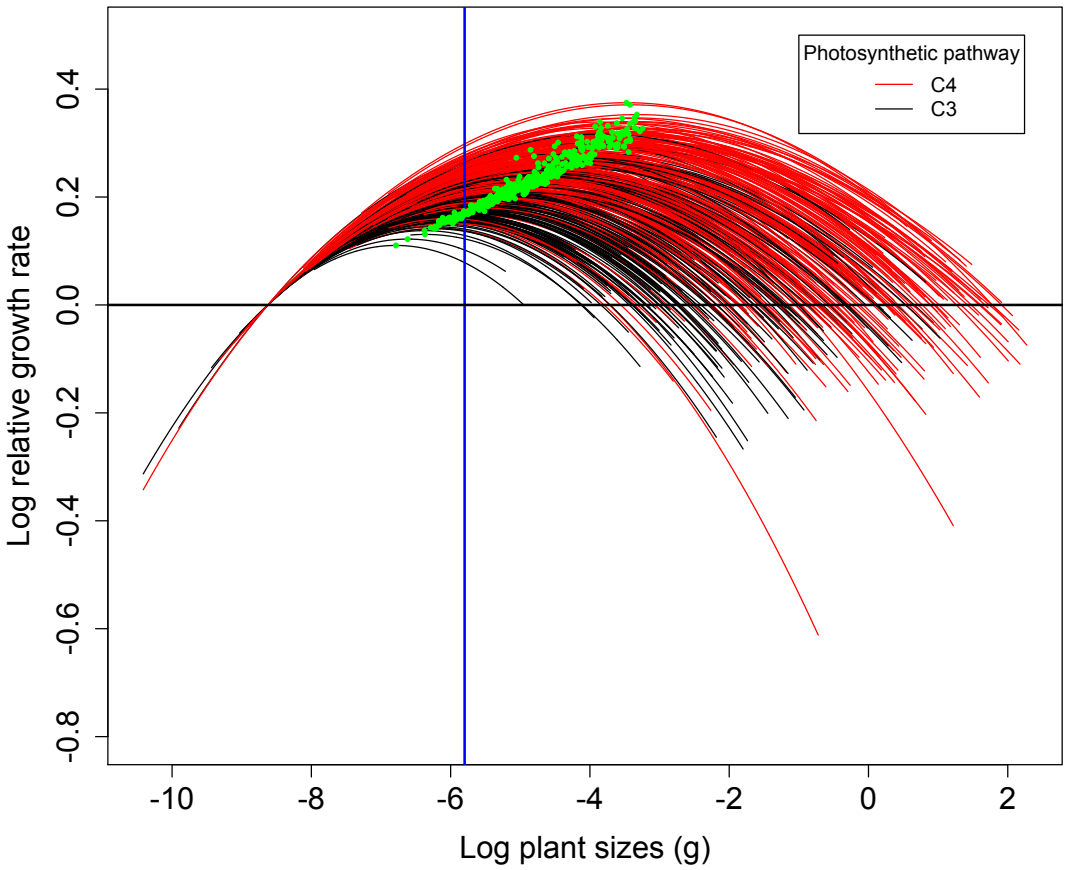
At small plant sizes, the contribution by NAR was negative overall because of its negative co-variance with SLA, so that a unit increase in NAR also decreased SLA, which overall resulted in a decrease in RGR. Over all species, NAR comprised -28% of the variation in RGR at small plant sizes (20% percentile). However, as plant size increased, so did the contribution of NAR to RGR variation growth (Supplementary Fig. 2). Overall, NAR was more important for growth in the C_3 than C_4 species, but this difference became less apparent at larger plant sizes (Supplementary Fig. 2), At large plant sizes, 83% of the variance in NAR was due to species-specific effects, while only 17% was due to the C_4 pathway.

Over all species, SLA comprised 126% of the variation in RGR at small sizes. Our experiment was completed at a high irradiance, and so the finding that SLA was important for fast growth could not be explained by poor light levels, which has been suggested previously[12]. At small plant sizes, the variation in SLA linked to photosynthetic pathway (i.e. the change in SLA that occurred on average from C_3 to C_4) was substantial, with 39% of variation due to the C_4 pathway, and 61% due to species-specific effects (Supplementary Table 2). We can also look at the contribution of SLA to RGR variation across clades and photosynthetic pathway types (Supplementary Fig. 3). This showed that SLA was more important for C_4 species than for C_3 species in the PACMAD or BEP clades. Particularly at small plant sizes, up to the median size, SLA made a larger contribution to RGR in the C_4 PACMAD than the C_3 species.

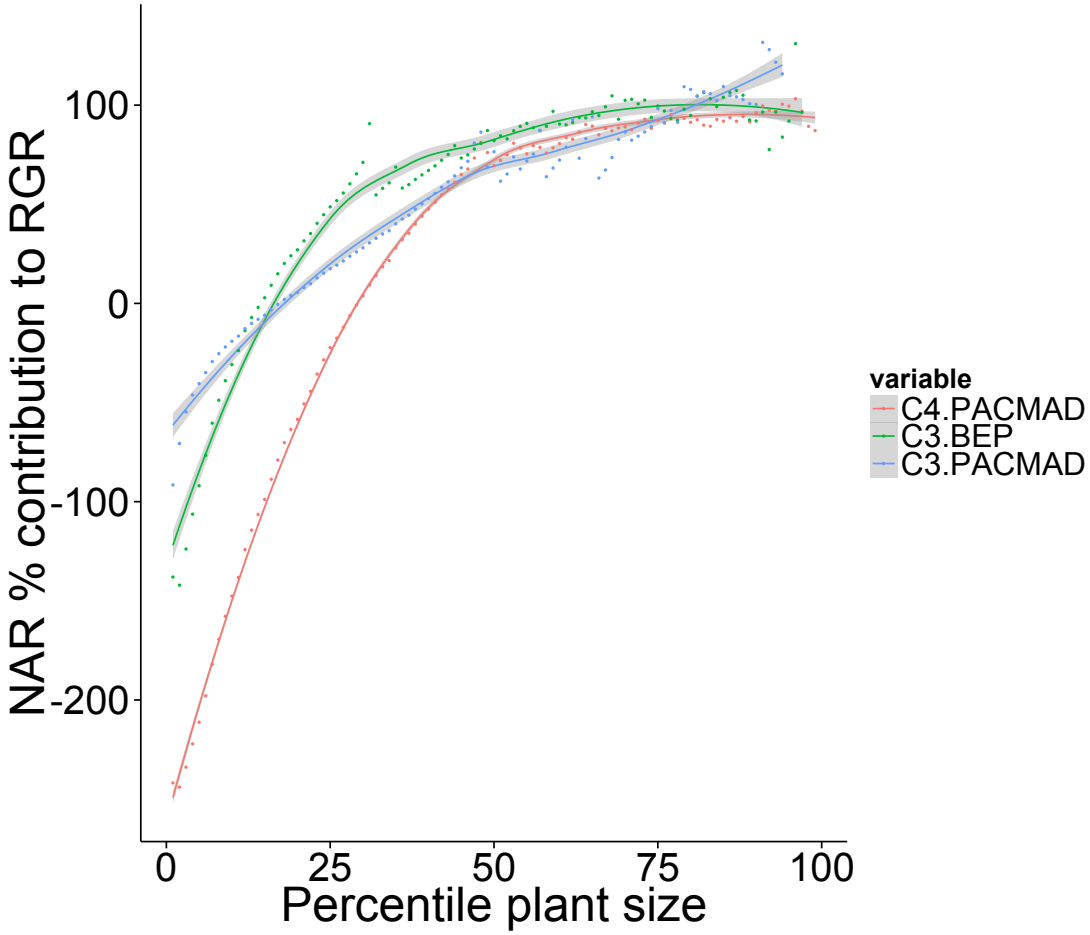
At the 20% size percentile, C_4 species had a 12% reduction in LMR compared with C_3 species, from 0.42 in the C_3 species to 0.37 in the C_4 species (df=1,380, t=3.46, λ=0.16, $p<0.001$) and LMR comprised 2% of the variation in RGR. At the 60th size percentile there was also a significant reduction in LMR, from 0.43 in the C_3 species to 0.34 in the C_4 species

(*df*=1,380, *t*=4.05, λ=0.44, p<0.001) and LMR determined -0.06% of the variation in RGR. Therefore, although there were differences in LMR between species with different photosynthetic pathways, LMR was not a significant driver of the variation in RGR. Instead, SLA and NAR contributed to RGR more significantly at small and large plant sizes, respectively.

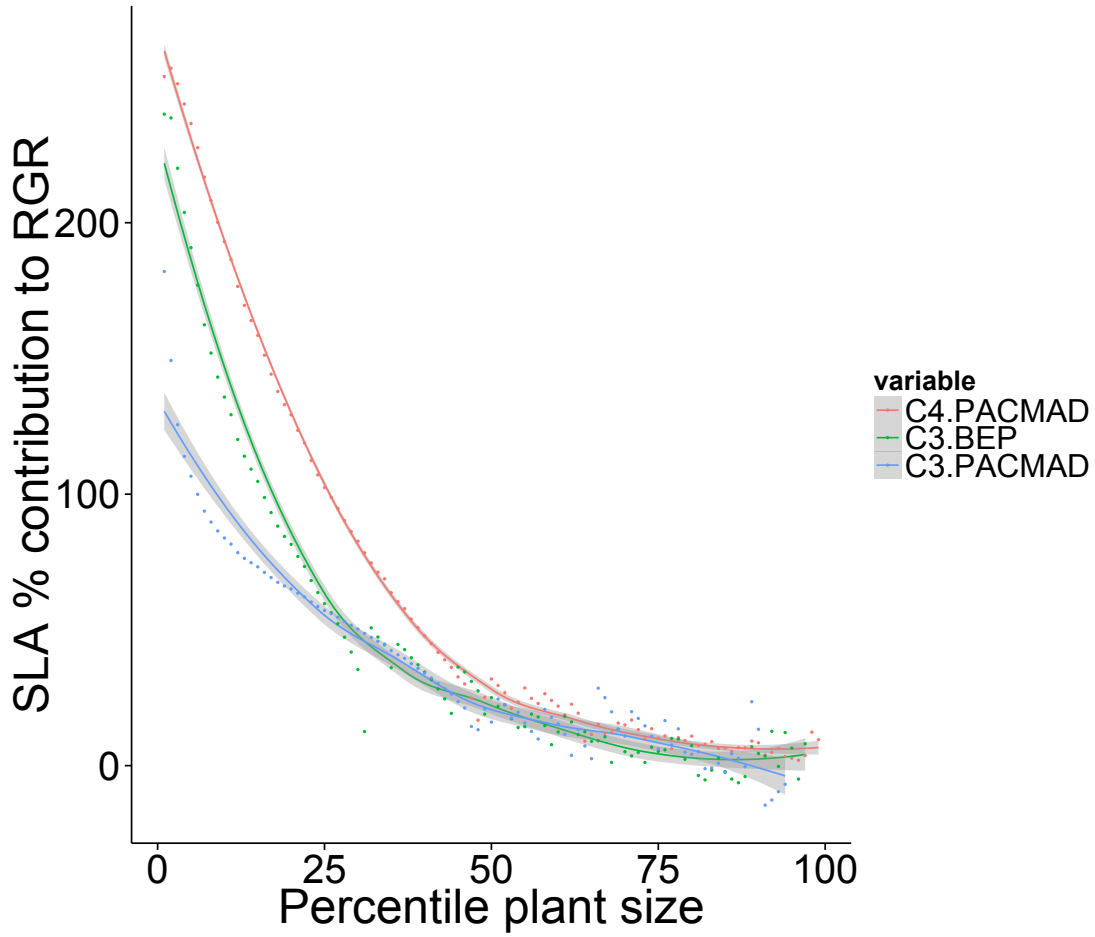
Supplementary Fig. 1 | Comparison of fitted growth rates across species in relation to size. The growth model used was the 4-parameter logistic fit, with each line representing a species. The green circles indicate the maximum growth rate for each species, and the blue vertical line shows the 20th percentile of the plant size distribution across all harvests. C_4 species on average had higher maximal growth rates, and reached larger final sizes.



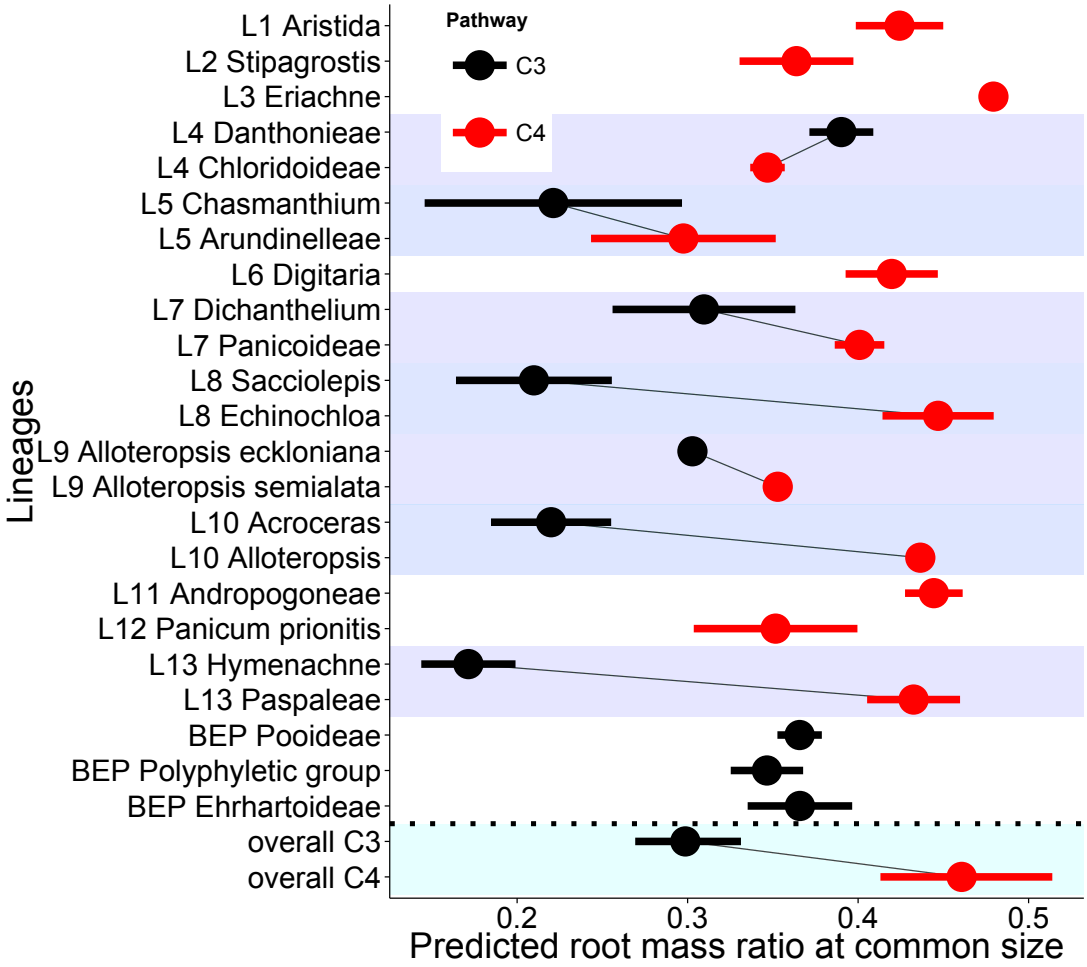
Supplementary Fig. 2 | Contribution of the interspecific variation in NAR to the variation in RGR. Percentage variation is shown for C_3 and C_4 PACMAD species and C_3 BEP species across the range of plant sizes.



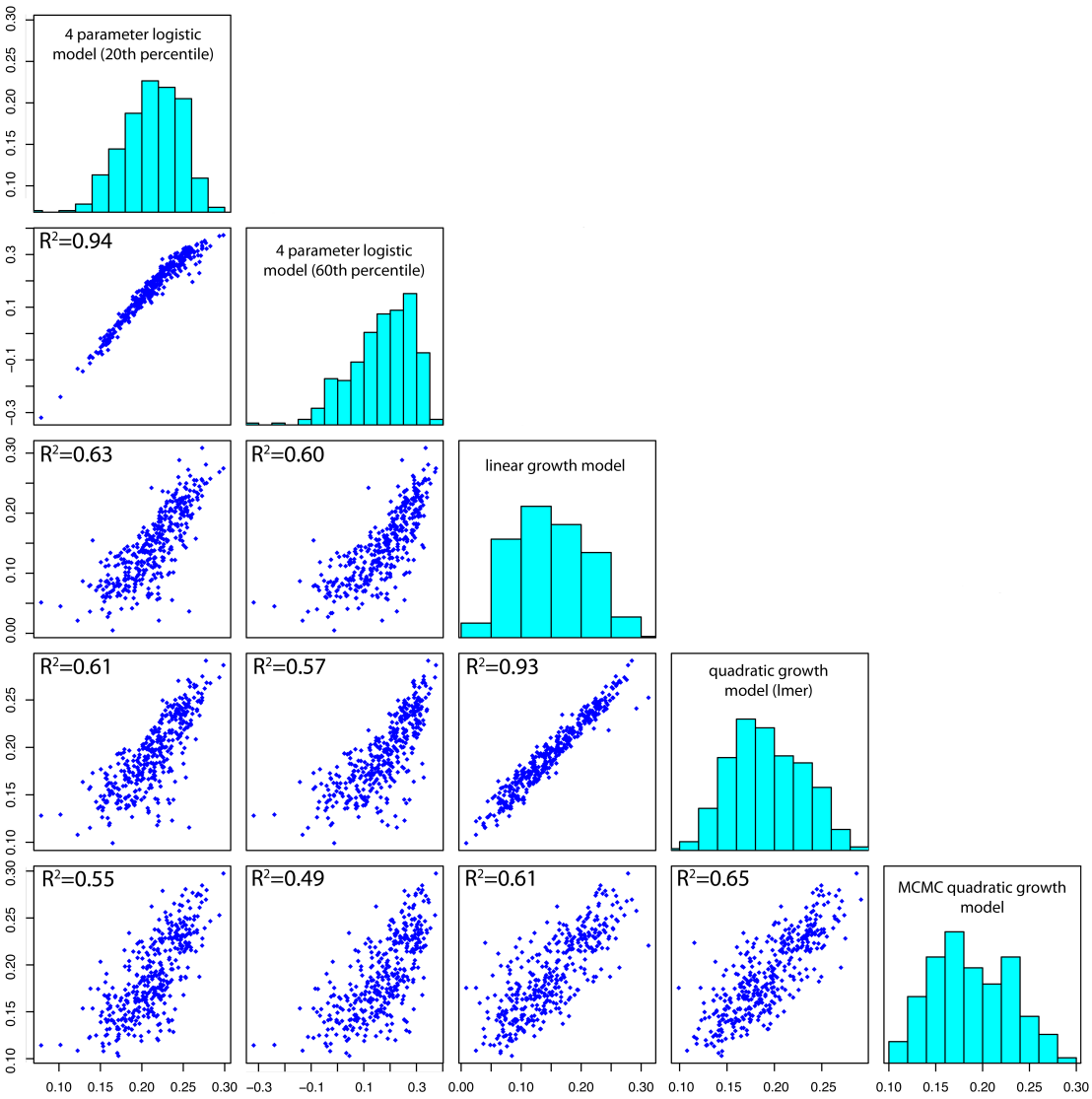
Supplementary Fig. 3 | Contribution of the interspecific variation in SLA to the variation in RGR. Percentage variation is shown for C_3 and C_4 PACMAD species and C_3 BEP species across the range of plant sizes.



Supplementary Fig. 4 | Root mass ratio at a common size. Root mass ratio (RMR) is the root dry mass as a fraction of total dry mass, and shown at the 20th percentile of the size distribution across all harvests. Comparisons between sister C_3 and C_4 lineages are highlighted by the linked bars, and the overall difference between C_3 and C_4 species calculated in a phylogenetic analysis is shown at the bottom of each panel. Means and standard errors for are depicted for C_4 lineages in red and C_3 lineages in black.



Supplementary Fig. 5 | Comparison of alternative approaches to modelling RGR. Relationships between the different RGR measures (below the main diagonal, all g g^{-1} d^{-1}), histograms of RGR calculated using each method (diagonal), and the R^2 for relationships between RGR values calculated by alternative methods.



Supplementary Table 1 | Species grown in the comparative experiment. Species are listed alongside the source of seed for each, the lineage they belong to (corresponding to Figs. 2, 3 and Supplementary Fig. 4), and the life history (annual / perennial), domestication status (W = wild, C = cultivar), growth form (s = sod-forming, n = not sod-forming), habitat preferences (see Supplementary Table 5), plant height in the field (culm height, cm), and climatic niche data (MAT, °C; MAP, mm), which were used as explanatory variables in the comparative analysis.

Table is available as Supplementary Table 1.csv

Supplementary Table 2 | Comparison of ANOVA results across different RGR models. In each case we tested the effects on RGR of climate (precipitation and temperature across the species range), domestication status, habitat (wetness and shadiness from floral descriptions), plant size at reproduction in the field (culm height), growth form, life history and photosynthetic type, whilst accounting for phylogeny (DF = degrees of freedom, s.error = standard error). Pagel's lambda indicates the extent to which the residual variation in RGR depends on phylogeny, according to a Brownian model of trait evolution. It varies between 0 and 1, with values of 0 implying no phylogenetic dependence, and values of 1 indicating perfect phylogenetic dependence. As sample size varied between species we allowed the error variance to be a power function of sample size (using the varPower function from the nlme library).

	Relative growth rate type										
	4 parameter logistic 20th percentile			4 parameter logistic 60th percentile		Linear growth model		Quadratic growth model (lmer)		MCMC quadratic growth model	
	DF	F-value	P-value	F-value	P-value	F-value	P-value	F-value	P-value	F-value	P-value
Intercept	1	5467.86	<.0001	294.84	<.0001	716.38	<.0001	1427.71	<.0001	615.07	<.0001
Precipitation	1	0.16	0.69	0.01	0.94	3.55	0.06	2.64	0.11	0.04	0.84
Temperature	1	7.22	0.01	12.36	<0.001	4.11	0.04	4.11	0.04	0.30	0.58
Domestication status	1	5.22	0.02	3.85	0.05	0.01	0.95	0.36	0.55	2.39	0.12
Habitat wetness	6	2.16	0.05	2.82	0.01	2.67	0.02	2.32	0.03	0.63	0.71
Habitat shadiness	4	1.17	0.32	3.33	0.01	0.73	0.57	0.73	0.57	0.76	0.55
Culm height	1	5.18	0.02	2.91	0.09	0.37	0.54	0.07	0.79	0.38	0.54
Growth form	1	11.71	<.001	0.40	0.53	2.22	0.14	1.91	0.17	1.88	0.17
Life history	1	57.45	<.0001	58.05	<.0001	27.27	<.0001	24.47	<.0001	29.15	<.0001
Photosynthetic type	1	37.24	<.0001	26.27	<.0001	27.51	<.0001	19.44	<.0001	10.69	<.01
Residual s.error		0.93		2.12		1.72		0.26		0.16	
Residual DF		350		312		350		350		350	
DF		368		330		368		368		368	
Pagel's lambda		0.48		0.32		0.29		0.33		0.90	

Supplementary Table 3 | Contributions to the interspecific variation in RGR. Contributions are shown from the three components of growth (SLA, NAR and LMR) at the 20th and 60th percentiles of the plant size distribution across all harvests. The contribution to variation in SLA, NAR, and LMR due to the C_4 pathway, and due to species-specific effects is also shown. Note that, due to covariance among SLA, NAR and LMR, overall contributions can exceed 1.0.

Size		SLA	NAR	LMR
20th percentile	**Overall variance contribution to RGR**	**1.25**	**-0.28**	**0.02**
	Variance among species	0.77	-0.08	0.07
	Effect of C_4 photosynthesis	0.49	-0.20	-0.05
	C_4 effect (C_4 effect/overall variance)	**0.39**	**0.70**	**-2.38**
60th percentile	**Overall variance contribution to RGR**	**0.24**	**0.82**	**-0.06**
	Variance among species	0.14	0.68	-0.02
	Effect of C_4 photosynthesis	0.10	0.14	-0.04
	C_4 effect (C_4 effect/overall variance)	**0.41**	**0.17**	**0.72**

Supplementary Table 4 | Contributions to the interspecific variation in SLA. Contributions are shown from the two main components of SLA (leaf thickness and leaf density) across all harvests. The contribution to variation in leaf thickness and leaf density due to the C_4 pathway, and due to species-specific effects is also shown. Note that, due to covariance among leaf thickness and leaf density, overall contributions can exceed 1.0.

	Leaf thickness	Leaf density
Overall variance contribution to SLA	**0.30**	**0.70**
Variance among species	0.28	0.52
Effect of C_4 photosynthesis	0.03	0.17
C_4 effect (C_4 effect/overall variance)	**0.09**	**0.25**

Supplementary Table 5 | Habitat coding scheme. Details of how habitat data was summarised for the a) wetness and b) shadiness categories for use in the comparative analyses of RGR.

a)

	Wetness category combination			
Final category level	Arid/dry	Damp	Wet	Waterlogged
arid	Y	N	N	N
dry	Y	Y	N	N
damp	N	Y	N	N
broad	N	N	N	N
broad	Y	Y	Y	N
broad	Y	Y	Y	Y
wet	N	Y	Y	N
wet	N	N	Y	N
very wet	N	N	Y	Y
very wet	N	Y	Y	Y
very wet	N	Y	N	Y
waterlogged	N	N	N	Y

b)

	Shadiness category combination		
Final category level	Open	Part shaded	Shady
Obligate open	Y	N	N
Not shady	Y	Y	N
Broad	Y	Y	Y
Broad	Y	N	Y
Broad	N	N	N
Obligate shade	N	N	Y

Supplementary Data 1 | Phylogenies for the species used in the experiment

The phylogenies are derived from a BEAST analysis. Two files are included in NEXUS format: the consensus tree (.tre) and a set of trees drawn from the posterior distribution (.trees).

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