Photosynthetic pathway and ecological adaptation explain stomatal trait diversity amongst grasses

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Summary

- The evolution of C₄ photosynthesis in plants has allowed the maintenance of high CO₂ assimilation rates despite lower stomatal conductances. This underpins the greater water-use efficiency in C₄ species and their tendency to occupy drier, more seasonal environments than their C₃ relatives.
- The basis of interspecific variation in maximum stomatal conductance to water (g_{max}), as defined by stomatal density and size, was investigated in a common-environment screening experiment. Stomatal traits were measured in 28 species from seven grass lineages, and comparative methods were used to test for predicted effects of C₃ and C₄ photosynthesis, annual precipitation and habitat wetness on g_{max}.
- Novel results were as follows: significant phylogenetic patterns exist in g_{max} and its determinants, stomatal size and stomatal density; C₄ species consistently have lower g_{max} than their C₃ relatives, associated with a shift towards smaller stomata at a given density. A direct relationship between g_{max} and precipitation was not supported. However, we confirmed associations between C₄ photosynthesis and lower precipitation, and showed steeper stomatal size–density relationships and higher g_{max} in wetter habitats.
- The observed relationships between stomatal patterning, photosynthetic pathway and habitat provide a clear example of the interplay between anatomical traits, physiological innovation and ecological adaptation in plants.

Introduction

The stomatal pores that perforate leaf surfaces are one of the best-characterized examples of the fundamental biological relationship between form and function (Hetherington & Woodward, 2003). The area and depth of each stomatal pore, together with the density of the stomata, determine the stomatal conductance to CO₂ and H₂O (Brown & Escombe, 1900; Parlange & Waggoner, 1970; Franks & Beerling, 2009a; Nobel, 2009), gaseous diffusion being regulated through turgor-mediated variation in the aperture of stomatal pores (Raschke, 1975; Buckley, 2005; Franks & Farquhar, 2007). The closure of stomata under dry atmospheric or soil conditions limits CO₂ diffusion from the atmosphere to chloroplasts, and means that stomatal physiology is inextricably linked to the physiology of photosynthesis (Farquhar & Sharkey, 1982). As a result, the patterning of stomata on leaf surfaces is correlated strongly with both hydrological conditions (Aasamaa et al., 2001; Sack et al., 2003; Franks et al., 2009) and photosynthetic capacity (Franks & Beerling, 2009a,b).

The evolution of the C₄ pathway has caused radical increases in potential photosynthetic capacity. The C₄ syndrome is one of the most important functional innovations in plants, and is particularly prevalent in grasses, where it occurs in c. 18 lineages and is utilized by around half of all modern species (Kellogg, 1999; Sage, 2004; Christin et al., 2008, 2009). The C₄ pathway operates as a CO₂-concentrating mechanism, elevating CO₂ concentrations locally around the carbon-fixing enzyme Rubisco, with the result that the rate of its carboxylase reaction is increased (Chollett & Ogren, 1975). In combination with the saturation of CO₂ to lower concentrations within leaf airspaces before photosynthesis is limited (Björkman, 1970; Bauwe, 1986). This, in turn, allows the same rate of photosynthesis to be maintained with a lower stomatal conductance in C₄ than C₃ leaves (Björkman, 1970; Long, 1999). Each evolutionary origin of C₄ photosynthesis from a C₃ ancestor might therefore be expected to present an opportunity for an associated reduction in the maximum stomatal conductance, providing water-use benefits over C₃ sister taxa. However, this hypothesis remains untested.

Recent comparative studies of grasses have indicated that C₄ photosynthesis is an adaptation to low atmospheric CO₂ (Christin et al., 2008; Vicentini et al., 2008) and open habitats (Osborne & Freckleton, 2009), evolving at high temperatures and permitting the colonization of drier, more seasonal subtropical environments.
Grasses exhibit further distinct traits relating to the efficiency and speed of guard cell movement (Franks & Farquhar, 2007), which are also thought to have facilitated adaptation to open environments (Hetherington & Woodward, 2003). However, the extent to which the diversity of stomatal traits among grasses is linked to habitat remains unknown.

We hypothesized that, across a diversity of independent evolutionary origins, C₄ grasses would consistently exhibit lower maximum stomatal conductance to H₂O ($g_{\text{max}}$) than C₃ grasses, associated with evolutionary shifts in stomatal patterning. Our recent work, which has emphasized the importance of controlling for phylogenetic diversity in comparisons of eco-physiological traits, has demonstrated that, on average, C₄ grasses across multiple lineages operate with lower stomatal conductance than species from C₃ sister lineages (Taylor et al., 2010, 2011). Here, we use comparative methods to address the following questions. Is C₄ photosynthesis associated with reduced $g_{\text{max}}$ compared with the C₃ type? Are differences in $g_{\text{max}}$ between species associated with precipitation or habitat water availability? Amongst grass lineages, do pore size and density, which determine $g_{\text{max}}$, show consistent patterns associated with photosynthetic type and ecological niche?

Materials and Methods

Species sampling and phylogeny

Species (Supporting Information Fig. S1) were sampled from C₄ and closely related C₃ lineages on the basis of phylogenetic information that was available in 2007 (Barker et al., 2001; Giussani et al., 2001; Aliscioni et al., 2003). Most groups included multiple species to allow for analysis within an ANOVA framework (Taylor et al., 2010). Here, we combined previously unpublished data on stomatal traits with a new phylogeny based on three markers were retrieved from GenBank and closely related C₃ lineages on the basis of phylogenetic information (Taylor et al., 2010). Under a general time-reversible substitution model with a gamma shape parameter and a proportion of invariant sites (GTR + G + I), two different analyses, each of four parallel chains, were run for 10,000,000 generations, sampling a tree every 1000th generation after a burn-in period of 3,000,000. A consensus tree was computed on the 14,000 sampled trees (Fig. S1).

Plant material and growing conditions

Plants were raised primarily from seed. Seeds were surface sterilized before germination on water agar, and then allowed to establish in plugs of compost (John Innes Seed Compost) before transplanting into 4-l pots of topsoil (Lawnmix topsoil®; Dandy’s Topsoil, Chester, UK). A minority of species were propagated vegetatively (Arundo donax, Arundo formosana, Hakonechloa macra) and transplanted directly into pots of topsoil. Plants were grown in a heated glasshouse in Sheffield, UK, between 21st May and 18th October 2007 (daily quantum input (mol m⁻² d⁻¹): mean, 9.7; maximum, 24.7; minimum, 1.9; relative humidity (%): daily mean 64; maximum, 92; minimum, 28; temperature (°C): daily mean, 20; maximum, 28; minimum, 15; recorded using a DL2e datalogger with RHT2nl and QS2 sensors; Delta-T Devices Ltd, Cambridge, UK). Species were randomized within eight blocks and plants were watered to saturation at least twice weekly. No supplementary nutrients were provided.

Measurement of stomatal traits

The youngest fully emerged leaf was removed at the ligule from one tiller of each plant in each experimental block. Leaves were taped onto sheets of newspaper to prevent curling, and allowed to air dry in a flower press. Dental putty (President Plus-light body; Coltène/Whaledent Ltd, Burgess Hill, West Sussex, UK) impressions were taken from the mid-section of both surfaces of the preserved leaves, and nail varnish peels produced from the impressions were transferred onto Polysine microscope slides (SLS; Hessle, North Humberside, UK). Stomatal guard cell length, pore length and pore density were measured using a microscope, camera and image processing equipment (Leitz Laborlux S; Leica Quantimet 500 running Quantimet 500 Q win software, Leica Microsystems (UK) Ltd, Milton Keynes, Buckinghamshire, UK; Sanyo CCD, SANYO Sales & Marketing Europe GmbH, Watford, Hertfordshire, UK). On each slide, along a diagonal transect of the peel, five stomata were measured for guard cell and pore lengths at 400x magnification. The stomatal density on each leaf surface was determined as the mean number of stomata visible in five 0.25-mm² fields of view, sampled along the diagonal of each peel.
Calculation of $g_{\text{max}}$

Maximum stomatal conductance to water vapour ($g_{\text{max}}$) was calculated as the sum of the maximum conductance values for each side of each leaf ($g_i + g_j$), based on the model of Brown & Escombe (1900) after Franks & Beerling (2009a). Alternative formulations of the Brown and Escombe model have been described by Weyers & Meidner (1990: pp. 56–57) (see also discussion in Franks & Farquhar, 2001). The equation for $g$ of one side of the leaf is

$$g_i = \frac{d}{v} \cdot D \cdot \frac{a_{\text{max}}}{l + \frac{v}{2} \sqrt{\frac{d_{\text{max}}}{\pi}}},$$

Eqn 1

where the subscript $i$ indicates the relative conductance to water vapour: $i = 1$ for the side of the leaf with the minimum value of $g$ and $i = 2$ for the side of the leaf with the maximum value of $g$. The diffusivity of water in air ($d$, m$^2$s$^{-1}$, at 25°C), the molar volume of air ($v$, m$^3$ mol$^{-1}$, at 25°C) and $\pi$ are physical and geometric constants. The stomatal density ($S$, mm$^{-2}$) was measured as described above. The stomatal size ($S = \text{guard cell length} \times \Sigma \text{guard cell widths}, m^2$) was calculated from our measurements of stomatal length. Following Franks & Beerling (2009a), we assumed that the depth of stomata ($l$, m) is equal to the guard cell width (i.e. guard cells are circular in cross-section). The maximum stomatal pore area ($a_{\text{max}}$, m$^2$) was predicted from its relationship with $S$, as measured from photomicrographs of fully open stomata on the leaves of 5-wk-old barley plants (grown in a greenhouse in 2-l pots of commercial compost and kept well watered). These had acclimated for several hours in full sun under water-saturated conditions. Leaf segments c. 3 cm in length were cut from mature leaves and placed directly onto the microscope stage. Within 2–3 min of excision, photomicrographs were collected using an inverted microscope equipped with a $\times 40$ long-working-distance objective (Diaphot 200; Nikon Instruments Europe B.V., Amstelveen, the Netherlands).

Leaf level values for $g_{\text{max}}$ were calculated as the sum of one-sided values for each leaf ($g_i + g_j$). The extent to which $g_{\text{max}}$ was dominated by a single side of the leaf was quantified by the ratio of the smallest to the largest of the one-sided values ($g_i : g_j$).

Characterization of hydrological niche

The realized precipitation niche of each species was described using geo-referenced species records obtained from the Global Biodiversity Information Facility (GBIF, http://www.gbif.org, accessed 26th September 2010). Species records were mapped onto 10’ grid squares defined within the Climate Research Unit CL 2.0 global climatology (New et al., 2002). Mean values for total annual precipitation, across the geographical range of each species, were calculated from precipitation values for 10’ grid cells in which each species occurred. To account for habitat-scale variation in the hydrological niche, we also compiled a list of habitats from species descriptions in regional floras (Clayton, 1970, 1989; Launt, 1971; Gibbs Russell et al., 1990; Western Australian Herbarium, 1998–; Cope, 1999, 2002; Van Oudtshoorn, 1999; Edgar & Connor, 2000; Malyschev & Peschkova, 2001; Tzvelev, 2001; Barkworth et al., 2003; Chen et al., 2006). We used these to classify species into two groups: those that were described explicitly as inhabiting wet habitats, for example, bogs, rivers, streams and water bodies (‘wet’), and those that were not (‘mesic-dry’).

Comparative methods

Analyses were carried out using species means, which were calculated from between two and eight replicates. To allow for the use of ANCOVA designs combining both discrete and continuous independent variables, we employed a phylogenetic generalized least-squares approach (PGLS, Grafen, 1989; Martins & Hansen, 1997). Correlation structures that accounted for phylogenetic covariance between species means were generated, based on pairwise shared distances on the phylogenetic tree, using Pagel’s $\lambda$ (Pagel, 1999; Freckleton et al., 2002; Freckleton, 2009). Optimum values of $\lambda$ were identified, and models were evaluated using a maximum likelihood modelling approach, implemented in R (Freckleton et al., 2002; pglnm3.3 code available on request from R. P. Freckleton, University of Sheffield, UK). Phylogenies were edited, and the phylogenetic covariance matrix was generated using the R package ape (Paradis et al., 2004). To evaluate the robustness of predictions to the comparative method used, for those models of stomatal traits in which a simple ANOVA design was applicable, an Ornstein–Uhlenbeck (OU) approach, implemented in the R package ouch (Butler & King, 2004; King & Butler, 2009), was used to generate independent estimates of mean trait values (Table S3). Selective regimes along our phylogeny, applied in the OU models, were estimated in R via maximum likelihood using the ace function in ape, selecting the best-fitting model from symmetrical, all-rates-different and equal-rates models on the basis of the Akaika information criterion (AIC). Pagel’s correlation analysis, as implemented in Mesquite (Maddison & Maddison, 2010), was used to test for independence in the evolution of pairs of discrete traits. The likelihood test statistic was computed on the basis of 30 initial likelihood searches and 1000 simulations.

Results

Precipitation and habitat

Precipitation niches were, on average, significantly drier for species with the $C_4$ photosynthetic type than those with $C_3$ (Fig. 1; Table 1, model A). This difference was not affected by the habitat occupied by each species, nor was there a significant difference in precipitation niches between species preferring mesic-dry vs wet habitats (Table 1, model A). In this model of precipitation niche as affected by photosynthetic type and habitat, the estimate of phylogenetic covariance (Pagel’s $\lambda$) was zero, that is, the modelled effects were independent of phylogenetic distance. Mean values for the precipitation niche of the $C_3$ and $C_4$ groups predicted by the PGLS model were highly consistent with optimum trait values obtained using the OU approach (± 6%
Fig. 1 Species values for precipitation niche by habitat type (wet, triangles; mesic-dry, circles) for C3 (closed symbols) and C4 (open symbols) grasses used in the screening experiment.

Table S3). The independent evolution of photosynthetic type and habitat preference along our phylogenetic tree was confirmed using Pagel’s 1994 test (difference in log-likelihoods, 0.27; P (traits independent) = 0.857). Contrasts between species of wet and mesic-dry habitats occurred within both C3 and C4 clades.

Stomatal patterning

The allometry of individual stomata in grasses, with their distinctive dumb-bell-shaped guard cells, was derived from a variety of published photomicrographs and scale drawings (Fig. 2). The width of grass stomata is approximately equal to 0.25 × stomatal length (Fig. 2a), whereas the guard cell width, and hence the pore depth (l), is approximately equal to 0.5 × stomatal width. We found that $a_{max}$ was approximately $0.4 \times S$ when measured for fully turgid barley leaves (Fig. 2b,c).

Because the degree of amphistomy varied between species, and some species had stomata on one side of their leaves only, we tested the effects of photosynthetic type, habitat and phylogeny on stomatal patterning by focusing on the side of the leaf that had the greatest calculated conductance capacity ($g_s$). An initial examination of differences in S and D indicated that species belonging to the Aristida and Chloridoideae clades tended to have smaller values of S than other C4 species (Fig. 3). By contrast, six of the seven species with $S > 300$ mm$^{-2}$ were members of the Paniceae tribe, and all of the species from the tribe Andropogoneae and subfamily Arundinoideae exhibited values for D that were greater than the median value for the dataset (Fig. 3).

Phylogenetic covariation in each of these stomatal patterning traits was supported by separate tests for the effects of photosynthetic type on log$_e$ D and log$_e$ S; in each case, there was evidence for a strong phylogenetic signal (Table 1, models B and C). After accounting for these phylogenetic effects, there was no significant difference in S between C3 and C4 species, but a significant 40% difference in the mean values of D (Table 1, model B) between C3 (mean, 173 mm$^{-2}$; SEM, 126–238 mm$^{-2}$) and C4 (mean, 124 mm$^{-2}$; SEM, 94–163 mm$^{-2}$) species. Although the OU method predicted a slightly larger difference in S than the PGLS method, parameter estimates for both S and D were comparable between the two methods (Table S3).

An inverse relationship, linearized by log transformation, is typically reported between S and D at the between-species level, and our data matched this expectation (Fig. 4a). After correction

### Table 1 Phylogenetic generalized least-squares models used to explore differences in precipitation and habitat classification between C3 and C4 species, and the influence of photosynthetic type, precipitation and habitat classification on stomatal traits

<table>
<thead>
<tr>
<th>Precipitation and habitat</th>
<th>(A) log$_e$ rain = 7.12 – 0.67 C4 – 0.17 wet + 0.25 C4 wet (AIC = 40.9)</th>
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<tbody>
<tr>
<td></td>
<td>$\lambda \approx 0, L_1 (\lambda_0 - \lambda_2) \approx 0, P = 1$</td>
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<tr>
<td></td>
<td>$F_{1,24} = 10.9, P = 0.003$</td>
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<tr>
<td></td>
<td>Wet = 0.03, 0.869</td>
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<td>C4 wet = 0.52, 0.479</td>
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<thead>
<tr>
<th>Stomatal patterning</th>
<th>(B) log$_e$ D = 5.16 – 0.34 C4 (AIC = 41.7)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\lambda = 0.86, L_1 (\lambda_{0.86} - \lambda_2) = 10.08, P = 0.002$</td>
</tr>
<tr>
<td></td>
<td>$F_{1,26} = 5.9, P = 0.023$</td>
</tr>
<tr>
<td></td>
<td>C4 = 5.18, 0.173</td>
</tr>
<tr>
<td></td>
<td>log$_e$ D = 2.54, 0.127</td>
</tr>
<tr>
<td></td>
<td>log$_e$ D wet = 3.08, 0.994</td>
</tr>
<tr>
<td></td>
<td>C4 wet = 0.15, 0.703</td>
</tr>
<tr>
<td></td>
<td>log$_e$ D C4 wet = 0.204, 0.656</td>
</tr>
</tbody>
</table>

|                           | (C) log$_e$ S = 5.32 – 0.05 C4 (AIC = 35.4)                                    |
|                           | $\lambda = 0.65, L_1 (\lambda_{0.65} - \lambda_2) = 6.49, P = 0.011$         |
|                           | $F_{1,26} = 3.8, P = 0.063$                                                   |
|                           | C4 = 5.18, 0.173                                                              |
|                           | log$_e$ D = 2.54, 0.127                                                       |
|                           | log$_e$ D wet = 3.08, 0.994                                                   |
|                           | C4 wet = 0.15, 0.703                                                          |
|                           | log$_e$ D C4 wet = 0.204, 0.656                                                |

|                           | (D) log$_e$ S = 7.90 – 0.47 log$_e$ D – 0.71 C4 + 2.96 wet + 0.07 log$_e$ D C4 – 0.54 log$_e$ D wet − 1.62 C4 wet + 0.32 log$_e$ D C4 wet (AIC = 23.4) |
|                           | $\lambda = 0.44, L_1 (\lambda_{0.44} - \lambda_2) = 2.56, P = 0.109$          |
|                           | $F_{1,26} = 17.27, P < 0.001$                                                 |
|                           | log$_e$ D = 18.33, 0.001                                                       |
|                           | C4 = 5.18, 0.173                                                              |
|                           | log$_e$ D = 2.29, 0.144                                                       |
|                           | log$_e$ D wet = 5.44, 0.029                                                   |

|                           | (E) log$_e$ S = 7.63 – 0.43 log$_e$ D – 0.34 C4 + 2.48 wet – 0.45 log$_e$ D C4 + 0.71 C4 + 2.96 wet (AIC = 18.9) |
|                           | $\lambda = 0.43, L_1 (\lambda_{0.43} - \lambda_2) = 3.50, P = 0.061$         |
|                           | $F_{1,26} = 19.79, P < 0.001$                                                 |
|                           | log$_e$ D = 18.33, 0.001                                                       |
|                           | C4 = 5.18, 0.173                                                              |
|                           | log$_e$ D = 2.29, 0.144                                                       |
|                           | log$_e$ D wet = 5.44, 0.029                                                   |

|                           | (F) log$_e$ S = 9.09 – 0.72 log$_e$ D – 1.63 C4 + 0.26 log$_e$ D C4 (AIC = 21.2) |
|                           | $\lambda = 0.58, L_1 (\lambda_{0.58} - \lambda_2) = 7.88, P = 0.005$          |
|                           | $F_{1,26} = 3.99, P = 0.057$                                                  |
|                           | log$_e$ D = 18.33, 0.001                                                       |
|                           | C4 = 5.18, 0.173                                                              |
|                           | log$_e$ D = 2.29, 0.144                                                       |

**Maximum leaf stomatal conductance (g$_{max}$)**

|                           | (G) log$_e$ g$_{max}$ = 0.59 – 0.33 C4 (AIC = 33.2)                           |
|                           | $\lambda = 0.70, L_1 (\lambda_{0.70} - \lambda_2) = 7.70, P = 0.006$         |
|                           | $F_{1,26} = 7.11, P = 0.013$                                                  |
|                           | C4 = 5.18, 0.173                                                              |

|                           | (H) log$_e$ g$_{max}$ = −0.42 – 0.0007 rain + 0.67 C4 + 1.73 wet – 0.0008 rain C4 − 0.0011 rain wet − 1.50 C4 wet + 0.0012 rain wet C4 (AIC = 34.8) |
|                           | $\lambda = 0.55, L_1 (\lambda_{0.55} - \lambda_2) = 4.53, P = 0.033$         |
|                           | $F_{1,26} = 2.90, P = 0.104$                                                  |
|                           | Rain = 4.45, 0.048                                                             |
|                           | C4 = 5.18, 0.173                                                              |
|                           | Wet = 5.15, 0.034                                                             |
|                           | Rain C4 = 0.146, 0.707                                                        |
for phylogenetic covariance, we found no significant interaction terms in a maximal model of \( \log_{e} S \) as a function of \( \log_{e} D \times \) photosynthetic type \( \times \) habitat (Table 1, model D). A minimal model, produced using AIC as a criterion for the stepwise exclusion of terms, indicated that habitat preference had a significant effect on the slope of the \( \log_{e} S \)-\( \log_{e} D \) relationship (Table 1, model E), which was shallower amongst species from mesic-dry environments (Fig. 4b). The photosynthetic type had no significant effect on the slope of the \( \log_{e} S \)-\( \log_{e} D \) relationship in either model, but there were significant differences in the intercept between C3 and C4 species in both cases (Table 1, models D and E). The minimal model suggested that, for species with high \( D \), habitat was relatively unimportant in determining \( S \), which differed primarily between C3 and C4 species (Fig. 4b). Amongst species with low \( D \) (first quartile, 114 mm \(^{-1} \)), habitat preference accounted for substantial differences in \( S \) predicted amongst C4 species from mesic-dry environments was 74% of that in wet environments, whereas, for C3 species from mesic-dry environments, predicted \( S \) was 67% of that in wet environments. Phylogenetic covariance was similar between the minimal and maximal models and did not have a significant impact on the fit of either model (Table 1, models D and E). The reduced importance of the phylogenetic covariance in these models, relative to those for the individual stomatal traits, may be a result of the strong influence of habitat on stomatal patterning. When habitat effects were not included in the initial model of \( \log_{e} S \)-\( \log_{e} D \times \) photosynthetic type, accounting for phylogenetic covariance significantly improved the model (Table 1, model F).

**Maximum leaf stomatal conductance (\( g_{\text{max}} \))**
The tendency for C4 species to show lower \( D \), and lower values of \( S \) for a given \( D \) on the side of the leaf with the greatest conductance value (\( g_{b} \)), suggested that C4 species should generally have lower values of \( g_{\text{max}} \) (\( g_{1} + g_{2} \)). A phylogenetically corrected model of \( \log_{e} g_{\text{max}} \times \) photosynthetic type confirmed this expectation, showing a significant difference between C3 and C4 species (Table 1, model G). The model predicted that, on average, \( g_{\text{max}} \) was 29% lower in C4 (mean, 1.29 mol m\(^{-2} \) s\(^{-1} \); SEM, 1.06–1.58 mol m\(^{-2} \) s\(^{-1} \)) than in C3 (mean, 1.80 mol m\(^{-2} \) s\(^{-1} \); SEM, 1.43–2.27 mol m\(^{-2} \) s\(^{-1} \)) species. The best-fitting value of \( \lambda \) for

<table>
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<th>Table 1 (Continued)</th>
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<tr>
<td></td>
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<tr>
<td>Rain wet</td>
</tr>
<tr>
<td>C4 wet</td>
</tr>
<tr>
<td>Rain C4 wet</td>
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</table>

(AIC = 33.1)

\( \lambda = 0.55, L(\lambda) = 4.18, P = 0.041 \)

\( F_{1,24} = p \)

C4 7.0 0.014

Wet 4.67 0.041

C4 wet 0.002 0.964

**Asymmetry between leaf surfaces (\( g_{1} : g_{2} \))**

\( \log_{e} g_{\text{max}} = 0.14 + 0.89 g_{1} : g_{2} + 0.00005 \)

\( C4 = 0.66 g_{1} : g_{2} C4 \) (AIC = 27.5)

\( \lambda = 0.81, L(\lambda) = 11.31, P < 0.001 \)

\( F_{1,24} = p \)

\( g_{1} : g_{2} \)

10.26 0.004

C4 4.88 0.037

\( g_{1} : g_{2} C4 \)

2.16 0.154

**Fig. 2** (a) Stomatal size (\( S \)), as defined by guard cell length (\( L \)) and width (\( W \)). Data are values for species, based on measurements from the following: line drawings in Metcalfe (1960; triangles, apex down); photomicrographs in Flint & Moreland (1946; circle) and Kaufman et al. (1970; triangle, apex up); images of stomata from Franks & Farquhar (2007; square); and photomicrographs of barley stomata (P. J. Franks, unpublished; diamond). Dotted line shows isoclines for different values of \( S \). Solid line shows the predicted relationship \( L = 3.5W + 5.0, \) estimated using least squares. Dashed line shows the simplified relationship, \( L = 4W, \) used for modelling purposes. (b) Relationship between pore area (\( A \)) and stomatal size (\( S \)) based on measurements from 13 images of stomata similar to (c). Solid line shows the predicted relationship \( a = 0.335 + 47.6, \) estimated using least squares. Dashed line shows the simplified relationship, \( a = 0.45, \) used for modelling purposes. (c) Photomicrograph of an open stomatal pore on an attached leaf of barley (P. J. Franks, unpublished).
this model was relatively high (0.70) and resulted in a significant improvement in model likelihood (Table 1, model G). Estimated optimum trait values for $g_{\text{max}}$ in an equivalent OU model were 15% and 20% higher, respectively, for C$_3$ and C$_4$ species, but fell within the estimated standard error of the means based on the PGLS model (Table S3).

For the log$_e$ $S$–log$_e$ $D$ relationship, we found that the phylogenetic dependence of $S$ and $D$ was diminished in importance when considering the effects of habitat. We therefore tested for the effects of habitat on $g_{\text{max}}$, and asked whether their inclusion in our models reduced the importance of phylogenetic covariance effects on model likelihood. When log$_e$ $g_{\text{max}}$ was modelled as a function of precipitation niche, photosynthetic type, and habitat, precipitation was not significant in explaining variance in $g_{\text{max}}$ (Table 1, model H). By contrast, and consistent with our analysis of the log$_e$ $S$–log$_e$ $D$ relationship, both photosynthetic type and habitat had significant and independent effects on $g_{\text{max}}$ (Table 1, model H). The estimated value of $z$ for this model was lower than that for the model without habitat (0.55), but, again, provided a significant improvement in model log-likelihood (Table 1, model H). The effects of C$_4$ photosynthesis and habitat classification on $g_{\text{max}}$ were therefore detected against a background of significant phylogenetic covariance in this trait.

As the precipitation niche was strongly dependent on the photosynthetic pathway (Fig. 1), we explored the relative effects of these two factors on $g_{\text{max}}$. On the basis of the AIC criterion, no terms could be dropped from our initial model (Table 1, model H), meaning that each factor had an effect on $g_{\text{max}}$ that could not be explained adequately by the other. When precipitation niche was excluded from the model, C$_4$ species had significantly lower $g_{\text{max}}$ values than C$_3$ species, and there was an increase in the $F$ value for photosynthetic pathway (Fig. 5; Table 1, model I). This suggests that the overall difference in $g_{\text{max}}$ between photosynthetic types may be partially explained by differences in precipitation niches between C$_3$ and C$_4$ species. The difference in $g_{\text{max}}$ attributed to photosynthetic type in the model excluding precipitation niche, remained independent of the significant difference in $g_{\text{max}}$ observed between species from wet and mesic-dry habitats (Fig. 5; Table 1, model I). Optimum trait values for $g_{\text{max}}$ from an OU model were consistently larger, but, again, within 20% of those predicted by the PGLS model (Table S3).
Asymmetry between leaf surfaces (g₁ : g₂)

The whole-leaf value of gmax comprises the sum of the predicted conductances for the two sides of the leaf (g₁ + g₂); therefore, the degree of amphistomy, that is, the equivalence in stomatal distribution/patterning between the sides of the leaf, measured here as g₁ : g₂, might be associated with gmax. If, for example, g₂ is similar between species, and g₁ varies, then g₁ : g₂ would be strongly associated with gmax. Alternatively, if increased g₁ was offset by a compensatory decrease in g₂, then gmax would be constant over the range of g₁ : g₂ from zero to unity. In the context of our comparisons, g₁ : g₂ might be associated with differences in gmax between photosynthetic types in two ways. First, either photosynthetic type might be more commonly associated with a specific range of g₁ : g₂ values. Second, if the range of g₁ : g₂ values is similar, an overall difference in gmax might result if the relationship between gmax and g₁ : g₂ differs between photosynthetic types. The median and range for g₁ : g₂ were similar amongst species within each photosynthetic type (C₃: median, 0.52; range, 0–0.82; C₄: median, 0.56; range, 0–0.91). However, although there were two species from each photosynthetic type with g₁ = 0, all of the remaining C₄ species (15/17, 88%) had g₁ : g₂ > 0.38, compared with just over one-half of the C₃ species (6/11, Fig. 6). Values of gmax for species with g₁ = 0 overlapped (Fig. 6) and, when loge gmax × photosynthetic type was re-tested with g₁ : g₂ included as a linear covariate, there was a substantial, but nonsignificant, shift in the slope of the loge gmax : g₁ : g₂ relationship between photosynthetic types (Table 1, model J), the slope being steeper amongst C₃ than C₄ grasses (Fig. 6). However, t-tests of coefficient values indicated that none of the coefficients for this model were significantly different from zero (t₁₇4 ≤ 1.53, P ≥ 0.501), perhaps as a result of the uneven distribution of C₄ species along the g₁ : g₂ axis. Although C₄ photosynthesis was clearly associated with an average reduction in gmax, this analysis provides some support for the hypothesis that the difference is greatest amongst species exhibiting greater degrees of amphistomy. As with the other models of gmax presented, correction for phylogenetic covariance provided a significant improvement in the fit of the model to the data (Table 1, model J).

Discussion

Our analyses support an adaptive hypothesis of stomatal evolution in grasses. First, the results indicate the correlated evolution of gmax and photosynthetic pathway. In keeping with previous work, our results also show that C₄ species tend to inhabit drier precipitation niches (Edwards & Still, 2008; Edwards & Smith, 2010). However, there is little evidence that gmax is influenced by precipitation niche independently of photosynthetic type. By accounting statistically for the effects of photosynthetic pathway, precipitation niche and habitat wetness, our analyses support a relationship between stomatal traits and the physiological contrast between C₃ and C₄ grasses.

Overall, it was found that gmax is lower in C₄ than in C₃ species, mirroring the previously reported lower operating leaf conductance observed for C₄ species (Taylor et al., 2010). This finding, of constitutive differences in gmax between C₃ and C₄ species, is consistent with well-established physiological differences between the two photosynthetic types. The role played by stomatal patterning as described by the S–D trade-off, in determining this difference, is also consistent with previous studies investigating the trade-off between CO₂ uptake and water loss (Hetherington & Woodward, 2003; Franks & Beering, 2009a; Franks et al., 2009).

The physiological trade-off between carbon fixation and water loss differs dramatically between C₃ and C₄ species (Björkman, 1970). This trade-off has driven adaptive shifts in S and D amongst C₃ species since the origins of terrestrial plants (Franks & Beering, 2009a). Overall, S and D are negatively correlated, such that higher gmax is associated with smaller S and higher D (Fig. 4; Franks & Beering, 2009a; Franks et al., 2009). However, reduced S and increased D can also lead to lower gmax if the reduction in S is sufficiently large, as observed for plants grown under treatment with the drought stress hormone abscisic acid (ABA) (Franks & Farquhar, 2001). The adaptation and evolution of gmax is therefore complex, and further work is necessary to
elucidate the drivers and evolutionary directions of the pattern in $S$, $D$ and $g_{\text{max}}$ observed in this study.

The effects of the photosynthetic pathway on $S$ and $D$ were on the margins of statistical significance, but the phylogenetic signal was strongly supported in the model of each trait. We inferred that adaptive changes in $g_{\text{max}}$ have resulted from various combinations of stomatal patterning traits, against a background of phylogenetic signals in $S$, $D$ and $g_{\text{max}}$. The nonsignificant difference in the response of $g_{\text{max}}$ to the degree of amphistomatomy observed between the photosynthetic types was also detected after correction for significant phylogenetic covariance. These results suggest constraints on the extent to which $S$, $D$ and, perhaps, $g_1 : g_2$ can vary within an individual lineage, and indicate that the proximate developmental mechanisms determining $g_{\text{max}}$ may depend critically on the phylogenetic group. Amongst C4 clades, for example, low $g_{\text{max}}$ in Aristida species is associated with low pore density, whereas, in Chloridoideae, it is associated with small pore size (Fig. 3). Based on these differences in trait values, it seems likely that the mechanistic underpinning of differences in $g_{\text{max}}$ is a further example of a similar functional outcome achieved through alternative evolutionary routes in different C4 lineages (Sinha & Kellogg, 1996; Kellogg, 1999; Christin et al., 2007, 2009).

More generally, it has been proposed that, whenever the CO2 supply becomes less limiting for photosynthesis, the high energetic costs of operating stomata should select against high $g_{\text{max}}$ in C4 species (Ueno et al., 2006), which is associated with the lower mesophyll to bundle sheath ratios diagnosing Kranz anatomy (Hattersley, 1984). As most stomata in grasses occur in rows between the vascular bundles (Metcalfe, 1960), the reduced distance between these in C4 species limits the proportion of the leaf surface area over which stomata can be distributed. It is interesting to note that, although not formally tested in this small dataset, the frequency with which C4 species showed a more even partitioning of $g_{\text{max}}$ between the two sides of the leaf was higher, a phenomenon which might arise as a result of physical constraints on the development of stomata on any one leaf surface.

Our analysis suggests that there are subtle differences in effect between photosynthetic pathway and habitat in their influence on stomatal traits. Independent of the effects of photosynthetic type, we found that $g_{\text{max}}$ was lower in species from dry-mesic habitats than in those from wet habitats. This is consistent with the hypothesis that stomatal patterning has evolved under selection from the degree of habitat wetness towards more or less conservative use of water. The interspecific pattern shown here, of a shallower distance between $S$ and $D$ amongst species from mesic-dry habitats when compared with those from wet habitats, replicates the results of a recent intraspecific study of the impacts of water availability on Eucalyptus (Franks et al., 2009). The similarity in the outcomes of these two studies is remarkable given the potential for impacts of gross leaf morphology, for example, architectural traits associated with leaf rolling (Redmann, 1985; Heathkorn & Delucia, 1991; Maricle et al., 2009), on stomatal patterning in comparisons of grass species from a variety of habitats.

The extent to which operational differences in leaf conductance between C3 and C4 species depend on the anatomy of stomatal patterning, as opposed to the physiological behaviour of stomatal aperture, which is considered to differ between C3 and C4 species (e.g. Jones, 1992), remains to be tested. However, our results indicate that evolutionary shifts in stomatal patterning comprise an important element in our understanding of the physiological impacts of the C4 syndrome.

Conclusions

We have shown that $g_{\text{max}}$, as determined by the size and density of stomata, is lower among C4 than among C3 grass species, a trend associated with a clear distinction between these photosynthetic types in terms of their precipitation niche. We have also shown that $g_{\text{max}}$ is lower in grass species from mesic-dry habitats than in those from wet habitats. Our results are consistent with the hypothesis that interspecific diversity in $g_{\text{max}}$ amongst grasses has arisen as a result of phylogenetic divergence in stomatal patterning, evolution of the C4 photosynthetic pathway and adaptation to habitat wetness. These results provide an excellent example of correlated evolution in physiological traits, showing how selection on physical form is mediated by physiological function.

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Supporting Information

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

Fig. S1 Consensus tree for 28 grass species based on three plastid markers: coding genes rbcL and ndhF; and the region encompassing trnK introns and the matK coding sequence.

Table S1 Primers used for the amplification of plastid markers

Table S2 Vouchers and accession numbers for the taxa used in the phylogenetic analyses

Table S3 Comparison of estimated mean trait values based on phylogenetic least squares with Pagel’s λ (PGLS λ, described in Table 1) and Ornstein–Uhlenbeck (OU) models

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