

Biophysical constraints on the origin of leaves inferred from the fossil record

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Edited by Robert A. Berner, Yale University, New Haven, CT, and approved June 1, 2004 (received for review April 20, 2004)

The molecular tool kit for producing flat-bladed photosynthetic structures evolved in marine and terrestrial plants during the middle Paleozoic, but it took a further 20 million years before leaves suddenly spread throughout land floras. This delay has long been difficult to explain, given the apparent advantage of leaves for photosynthetic primary production. Theory and experiments predict that exceptionally high atmospheric CO₂ levels in the middle Paleozoic delayed the origin of leaves by restricting stomatal development. This would have limited evaporative cooling, leading to lethal overheating of leaves absorbing large quantities of solar energy. Here we test the central prediction of this argument with a morphometric analysis of 300 plant fossils from major European collections. We show a 25-fold enlargement of leaf blades in two phylogenetically independent clades as atmospheric CO₂ levels fell during the late Paleozoic. Furthermore, preliminary data suggest that the first abrupt increase in leaf size was accompanied by an 8-fold rise in stomatal density. These evolutionary patterns support the relaxation of biophysical constraints on leaf area predicted by theory and point to a significant role for CO₂ in plant evolution.

The origin of megaphyll leaves was a major event in vascular land plant evolutionary history that proceeded through the developmental modification of lateral branch systems (1, 2). However, leaves did not become widespread in fossil floras until 50 million years after the emergence of vascular plants (3–5). This inordinately long delay is genuinely puzzling. Marine algae developed laminate photosynthetic organs some 10 mm wide as early as the Ordovician (6), and tiny megaphyll leaves are known from the rare Early Devonian vascular land plant *Eophyllophyton bellum* (7). Fossil evidence therefore demonstrates that the genes required for laminate structures had already evolved in independent groups, and that no intrinsic genetic or developmental barriers delayed the widespread appearance of leaves. Theory suggests an environmental barrier to leaf evolution, involving evaporative and convective cooling, leaf size, and CO₂-mediated stomatal evolution (8). This mechanism draws attention to the fact that low stomatal frequencies of early land plants (9, 10) in the high CO₂ atmosphere of the middle Paleozoic (Fig. 1A) placed a tight physical limit on evaporative energy loss (8). Large leaves developing in these plants would therefore have been prone to lethal overheating because of greater interception of solar energy and lower convective heat dissipation than a leafless stem (8). As atmospheric CO₂ levels subsequently declined (11) (Fig. 1A), stomatal numbers in land plants rose (9), and greater evaporative cooling ensued, alleviating the requirement for convective heat loss. Because the capacity for convective heat loss decreases with increasing leaf size, it follows that leaves became progressively larger as stomatal numbers rose and atmospheric CO₂ declined in the late Paleozoic, a prediction that can be tested against observations from the fossil record.

We tested this theoretical linkage between plant and atmospheric evolution on geological time scales by compiling and analyzing an extensive dataset of 300 leaf fossil species from Devonian and Mississippian floras. Trends in leaf size were investigated by measuring maximum width and the square root

of the lamina area, taken as an integrative measure of average leaf width. These dimensions together characterize the path length of air flow across the leaf, which is a major determinant of the boundary layer resistance to convective heat loss (12). The principal European fossil collections for the time interval of interest are highly influenced by coal exploration and mining and are therefore biased toward the paleotropics. However, rare specimens from higher paleolatitudes were included, adding a global dimension to the database. These allowed an assessment of the effects of a lower sun angle and cooler temperatures on leaf size.

Methods

Sampling for Devonian and Mississippian leaf fossils was extensive and designed to include well-preserved specimens for a wide range of species and collection localities (see Table 1, which is published as supporting information on the PNAS web site, for full details). The specimens were archived in European museum and university collections at the British Geological Survey (Keyworth, U.K.), Cardiff University (Cardiff, U.K.), National Museums of Scotland (Edinburgh), Natural History Museum (London), National Museum of Wales (Cardiff, U.K.), Royal Belgian Institute of Natural Sciences (Brussels), Senckenberg Institute (Frankfurt), Swedish Museum of Natural History (Stockholm), and University of Liège (Liège, Belgium). Our analysis excluded measurements for the rare morpho-taxa of detached fan-shaped leaves assigned to the artificial Order Palaeophyllales (13). The taxonomic affinity and ecology of these leaves are unknown (14), and their status as vascular plants remains uncertain in many cases, with the potential for confusion with laminate algae.

Measurements of the maximum leaf width and lamina area were made from high-resolution digital photographs of each specimen (Coolpix 995, Nikon), using image analysis and morphometrics software (TPSDIG, Ver. 1.39; F. James Rohlf, Morphometrics, State University of New York, Stony Brook). Where important specimens of fossil species were not represented in museum collections, measurements were made by using published photographs or *camera lucida* drawings of fossils but not from interpretive reconstructions of the fossil plant. Maximum leaf width was defined as the largest intact dimension of the lamina or terminal nonlaminar branch, measured perpendicular to the rachis. The lamina corresponded to a pinnule in the case of pinnate fronds and the entire leaf in simple (nonpinnate) forms. The area was determined only for intact laminae with a maximum width >5 mm. The square root of lamina area is a measure of the path length for air flow over the leaf in any direction.

Cuticles are preserved very rarely in Devonian fossils of the earliest laminate leaves. However, cuticle preparations were examined for *Archaeopteris macilentata* (15), together with isolated

This paper was submitted directly (Track II) to the PNAS office.

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spheric change, with stomatal characters fixed at values typical for early leafless land plants.

Results and Discussion

Our analyses indicate a clear trend of rising maximum (Fig. 1D) and average (Fig. 1E) megaphyll leaf width between 380 and 340 million years ago that broadly coincides with the drop in atmospheric CO₂ (Fig. 1A) and cooling of tropical temperatures (Fig. 1B and C). These temporal trends show that leaf blades became longer and broader, not simply less dissected, and are the result of two distinct phases of megaphyll evolution. The first widespread appearance of leaves occurs in the Progymnosperms during the Mid-Late Devonian (Fig. 1D and E), with the evolutionary radiation of *Archaeopteris* (e.g., Fig. 2A and B). Laminae become progressively larger through this interval (Fig. 1D and E), reaching maximum widths of 5 mm in the Givetian (e.g., Fig. 2A) and 39 mm in the Frasnian (e.g., Fig. 2B). This initial radiation of megaphylls is soon followed by a phylogenetically independent (5) origin and diversification of leaves within the Pteridosperms (e.g., Fig. 2C) during the Famennian and Mississippian (Fig. 1D and E). The Pteridosperms are characterized by large multipinnate megaphylls (“fronds”), and the leaflets of this group are significantly larger in our analysis than the older Progymnosperm leaves, attaining a maximum width of 77 mm (Fig. 1D) only after atmospheric CO₂ fell to its lowest level (Fig. 1A). Overall, this pattern of change argues for a biophysical rather than a developmental or phylogenetic driver of the trend.

Until now, stomatal frequencies have not been reported for leaves during their initial radiation, making it difficult to establish a direct linkage between leaf size and the potential for evaporative cooling. However, our examination of fossil cuticles (see *Methods*) belonging to two Frasnian *Archaeopteris* species (15, 16) shows stomatal densities (*A. macilenta* 32.0 ± 0.8 mm⁻² and cf. *Archaeopteris* 36.6 ± 3.0 mm⁻²) eight times greater than those of axial Early Devonian plants (4.5 ± 0.4 mm⁻²) (9, 10). The rise in density accompanied a 20-fold increase in the width of the photosynthetic organ (Fig. 1D). Moreover, the guard cells of *Archaeopteris* were large (62 ± 3 μm in *A. macilenta* and 57 ± 4 μm in cf. *Archaeopteris*), approaching the maximum observed in any modern or fossil plants (17). These evolutionary shifts in stomatal anatomy are consistent with the effects of rising CO₂ on modern land plants (18) and would have lowered the resistance of fully open stomata to water vapor (see *Methods*) by >10 times compared with their axial ancestors (12).

The major Late Paleozoic decline in atmospheric CO₂ levels was accompanied by significant climatic cooling (19), which

partially alleviated the risk of overheating in large primitive leaves. In a mathematical analysis, we isolated and quantified the relative roles of climate and stomata by simulating changes in leaf energy balance (8, 20) and either varying or holding constant stomatal characteristics and climate. The analysis showed that cooling without changes in stomatal density allowed maximum leaf size to increase by only three times (Fig. 1D). Intriguingly, measurements of leaf size for scarce *Archaeopteris* fossils from two high paleolatitude Devonian localities in South America (21) support this result. These show maximum widths ≈30% larger than low latitude plants of equivalent age (Fig. 1D). Repeating the energy balance calculations with the observed increases in stomatal numbers allowed simulated tropical leaf size to rise 25 times, in agreement with the fossil evidence (Fig. 1D and E). Our analysis therefore indicates that stomatal density plays a much greater relative role than climatic cooling in allowing progressive evolution of larger leaves.

We have shown that the origin and subsequent diversification of leaves tracks independent evidence for declining atmospheric CO₂ levels. The observed trends are entirely consistent with the relaxation of an energetic constraint mediated by CO₂-driven stomatal evolution. However, we recognize that once flat leaves had evolved, in concert with root and vascular systems (22, 23), they would have quickly propagated throughout terrestrial ecosystems by conferring significant selective benefits in competition for light and space. This competition is manifested in the fossil record by the well-documented evolution of plant height during the same interval (22). Viewed in this context, declining CO₂ levels may have lifted a barrier to the evolution of leaves, much as rising O₂ may have done for the Cambrian invertebrate explosion (24).

This work was supported by a Leverhulme Trust research grant (to D.J.B. and W.G.C.) and by Royal Society University Research Fellowships (to C.P.O. and D.J.B.). We thank P. Kenrick (Natural History Museum, London), V. Wilde (Senckenberg Institute, Frankfurt), M. Howe and P. Taylor (British Geological Survey, Nottingham, U.K.), E. M. Friis and Th. Denk (Swedish Museum of Natural History, Stockholm), C. Berry (Cardiff University, Cardiff, U.K.), M. Fairon-Demaret and P. Gerienne (University of Liège, Liège, Belgium), F. Damblon (Royal Belgian Institute of Natural Sciences, Brussels), and J. Hilton (National Museums of Scotland, Edinburgh) for kind assistance and access to fossil collections. We thank K. Niklas, M. A. Gandolfo (Cornell University, Ithaca, NY), and P. G. Gensel (University of North Carolina, Chapel Hill) for loans of fossil cuticles; D. Royer, C. Wellman, and M. C. Press for comments on the manuscript; and F. J. Rohlff for advice on morphometric analyses.

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