Commentary

What lies between: the evolution of stomatal traits on the road to C$_4$ photosynthesis

'When do changes in stomatal traits occur as a lineage evolves from an ancestral C$_3$ state towards full C$_4$ physiology?'

Photosynthesis evolved early in the history of life (Blackenship, 2010), and despite the ubiquity and importance of biological carbon fixation, the process is still far from optimal. The majority of the world's plant species perform C$_3$ photosynthesis, whereby CO$_2$ is initially fixed by the enzyme Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase). However, Rubisco can also react with O$_2$, leading to photorespiration, a process which consumes energy and releases previously fixed CO$_2$. The cost of photorespiration, an inhibition of up to 40% of photosynthesis in today's atmosphere, is thought to have been the driving force behind the evolution of C$_4$ photosynthesis (Sage, 2004; Gowik & Westhoff, 2011). Species that perform C$_4$ photosynthesis concentrate CO$_2$ around Rubisco, thereby greatly enhancing its carboxylation efficiency and largely eliminating photorespiration. This translates into high productivity, and C$_4$ species constitute some of our most successful crops, including maize (*Zea mays*) and sugarcane (*Saccharum officinarum*), as well our most promising biofuel species, such as switchgrass (*Panicum virgatum*) and *Miscanthus × giganteus*. Because the C$_4$ photosynthetic pathway has evolved over 60 times in at least 19 families (Sage *et al.*, 2011), the multitude of closely related C$_3$ and C$_4$ species provide a powerful tool for understanding the repeated evolution of C$_4$-associated traits. Many studies focus on comparing characteristics between C$_3$ and C$_4$ species in a single lineage, raising the issue of whether traits associated with C$_4$ species are truly C$_4$-related or are due to common evolutionary histories or habitat preferences (Edwards & Still, 2008). In this issue of *New Phytologist*, Taylor *et al.* (pp. 387–396) assess differences in stomatal characteristics in a suite of related C$_3$ and C$_4$ grasses. The authors show that stomatal traits vary predictably between C$_3$ and C$_4$ species, even when phylogeny and growth environment are accounted for in the analysis, thereby clearly attributing differences to functional convergence based on photosynthetic pathway. This work makes a novel contribution to our knowledge of C$_4$ biology and provides a hitherto missing link between stomatal characteristics and photosynthetic physiology.

In leaves, the uptake of CO$_2$ is inextricably linked to the loss of water through stomata, with an average of *c.* 2.7 g of carbon fixed per kilogram of water transpired in C$_3$ plants under non-stressful conditions. Because of this inherent trade-off, the regulation of stomatal conductance can be viewed as an optimization problem, whereby carbon gain per unit water loss is maximized (Cowan & Farquhar, 1977). Accordingly, since C$_4$ species have high photosynthetic rates even at low intercellular CO$_2$ concentrations, they should maintain lower stomatal conductance rates than C$_3$ species to reduce their transpiration rate and further increase their water-use efficiency (the ratio of photosynthesis to transpiration). Indeed, stomatal conductance rates are reduced in C$_4$ species (Taylor *et al.*, 2010), and C$_4$ plants have higher water-use efficiency than C$_3$ species (Monson, 1989; Sage, 2004; Vogan & Sage, 2011). Consistent with this earlier work, Taylor *et al.* demonstrate that even in a phylogenetically-controlled analysis, C$_4$ grasses have lower maximum stomatal conductance rates (g_{max}) than their C$_3$ relatives. However, the lower g_{max} of C$_4$ plants compared to C$_3$ species might still be due to differences in habitat (Edwards & Still, 2008). Photorespiration is enhanced at high temperatures and low intercellular CO$_2$ concentrations, and C$_4$ species tend to grow in hot and arid environments where photorespiratory costs are high (Sage, 2004). Since species in dry environments should restrict water loss regardless of their photosynthetic pathway, Taylor *et al.* also looked at precipitation niche to determine if differences in g_{max} were explained by water availability. In both mesic and arid environments, the authors found that even when accounting for phylogeny, C$_4$ species had lower g_{max} than C$_3$ species, demonstrating intrinsic differences in stomatal traits between the two groups. Generally, low g_{max} was achieved in C$_4$ plants by producing smaller stomata for a given stomatal density, although the authors found differences between lineages, such that some C$_4$ lines reduced stomatal density, while others preferentially reduced stomatal aperture. Evolutionary convergence towards a common functional solution, but using various anatomical or biochemical means, is a repeated theme in C$_4$ evolution (Sage, 2004), and this work shows that the same holds true for stomata. These results demonstrate the importance of incorporating both phylogenetic and environmental data into analyses of C$_4$ trait evolution, as well as filling in a crucial gap in our knowledge of C$_4$ stomatal characteristics.

When do stomatal characteristics change during C$_4$ evolution?

While Taylor *et al.* concentrated on C$_3$ and C$_4$ monocot species, their work raises interesting questions about the coupled evolution of photosynthetic biochemistry and stomatal characteristics in general. For example, when do changes in stomatal traits, such

Photosynthetic type

	C_3	Type I	Type II	C_4-like	C_4
			C_3–C_4 Intermediates		
Carbon-related traits					
Enlarged bundle sheath cells	✗	✓	✓	✓	✓
Photorespiratory CCM	✗	✓	✓	✓	✓
Increased PEPCase activity	✗	✗	✓	✓	✓
Active C_4 cycle	✗	✗	✗	✓	✓
Mesophyll Rubisco activity	✓	✓	✓	✓	✗
Stomatal-related traits					
Increased vein density	✗	✓	✓	✓✓	✓✓
Increased water-use efficiency	✗	✗	✗	✓	✓
Stomata regulate lower C_i	✗	✗	✗	✓	✓
Reduced stomatal density/size	✗	?	?	?	✓

Fig. 1 Key traits involved in the evolution of C_4 photosynthesis, with regard to the ancestral C_3 state. Although there is overlap in the categories, traits primarily associated with the development of an optimized C_4 cycle are in blue and traits that emphasize potential changes in stomatal characteristics are in green. While there is no data on when stomatal density or size are altered, changes in other traits related to stomatal regulation between Type II intermediates and C_4-like intermediates imply that stomatal development may also be affected at this transition. PEPCase, phosphoenolpyruvate carboxylase; CCM, carbon-concentrating mechanism; C_i, intercellular CO_2 concentration.

as stomatal density or size, occur as a lineage evolves from an ancestral C_3 state towards full C_4 physiology? In C_4 species, Rubisco is localized to the enlarged bundle sheath cells; phosphoenolpyruvate carboxylase (PEPCase) acts as part of the C_4 cycle which pumps organic acids from the mesophyll into the bundle sheath, where they are decarboxylated (Sage, 2004). In some genera, such as the eudicots *Flaveria* and *Heliotropium*, there are not only C_3 and C_4 species, but also C_3–C_4 intermediate species with varying levels of C_4 anatomy and physiology (Kocacinar *et al.*, 2008; Gowik & Westhoff, 2011; Muhaidat *et al.*, 2011; Vogan & Sage, 2011). Three main groups of intermediates are recognized, each a more derived state than the previous: in Type I intermediates, Rubisco refixes photorespiratory CO_2 in enlarged bundle sheath cells; Type II intermediates also have increased PEP carboxylase activity, indicating some level of a C_4 cycle; and lastly, C_4-like intermediates have a C_4 cycle to concentrate CO_2 in the bundle sheath, but still retain some residual Rubisco activity in the mesophyll (Fig. 1; Sage, 2004; McKown & Dengler, 2007; Kocacinar *et al.*, 2008; Gowik & Westhoff, 2011; Vogan & Sage, 2011).

In lineages such as *Flaveria* where phylogenetic data indicate that C_3–C_4 intermediacy occurs between the ancestral C_3 and the derived C_4 states, intermediates could provide a unique system for studying changes in stomatal development along a gradient of C_4 expression. *Flaveria* has already been used to study stomatal behavior to light and intercellular CO_2 concentrations in C_3, C_3–C_4 intermediates and C_4 species, as well as differences in water-use efficiency (Monson, 1989; Huxman & Monson, 2003; Vogan & Sage, 2011). Water-use efficiency is not enhanced in Type I or II *Flaveria*, or in *Panicum millioides*, a monocot intermediate which lacks a well-developed C_4 cycle (Monson, 1989; Pinto *et al.*, 2011; Vogan & Sage, 2011). As well, C_4 and C_4-like *Flaveria* species regulate stomatal conductance to maintain a lower intercellular CO_2 concentration than more C_3-like intermediates (Vogan & Sage, 2011), implying that changes in stomatal behavior arise late in the evolution of C_4 photosynthesis

(Sage, 2004). Could changes in stomatal anatomy and density also be most pronounced at the transition from Type II intermediacy to a C_4-like state (Fig. 1)? Part of the answer may lie in changes in leaf venation: high vein density appears to be a prerequisite for evolving C_4 photosynthesis in leaves (Sage, 2004), and since stomata generally develop between veins, this may provide a direct mechanism for limiting stomatal density. While C_4-like *Flaveria* species have higher vein density than either Type I or II intermediates (McKown & Dengler, 2007), increases in vein density also occur at the initial transition from C_3 to a Type I intermediate (Sage, 2004; McKown & Dengler, 2007; Gowik & Westhoff, 2011), so venation might constrain stomatal development much earlier in the path towards C_4.

The integration of structure and function has led to exciting insights into C_4 evolution, including links between photosynthetic pathway and hydraulic traits (Kocacinar *et al.*, 2008). The work by Taylor *et al.* highlights that not only are stomata functionally affected by the evolution of C_4 photosynthesis, but that these different functional responses may be underlain by structural changes. Their results also emphasize the need to consider evolutionary history and habitat when attributing traits to convergent evolution. Future research into the generalization of these results in C_4 eudicot lineages with C_3–C_4 intermediates will provide insight on the evolutionary pressures and constraints on stomata and carbon–water trade-offs along the path to C_4.

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