Increasing water use efficiency along the C₃ to C₄ evolutionary pathway: a stomatal optimization perspective

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Abstract

C₄ photosynthesis evolved independently numerous times, probably in response to declining atmospheric CO₂ concentrations, but also to high temperatures and aridity, which enhance water losses through transpiration. Here, the environmental factors controlling stomatal behaviour of leaf-level carbon and water exchange were examined across the evolutionary continuum from C₃ to C₄ photosynthesis at current (400 μmol mol⁻¹) and low (280 μmol mol⁻¹) atmospheric CO₂ conditions. To this aim, a stomatal optimization model was further developed to describe the evolutionary continuum from C₃ to C₄ species within a unified framework. Data on C₃, three categories of C₃–C₄ intermediates, and C₄ Flaveria species were used to parameterize the stomatal model, including parameters for the marginal water use efficiency and the efficiency of the CO₂-concentrating mechanism (or C₄ pump); these two parameters are interpreted as traits reflecting the stomatal and photosynthetic adjustments during the C₃ to C₄ transformation. Neither the marginal water use efficiency nor the C₄ pump strength changed significantly from C₃ to early C₃–C₄ intermediate stages, but both traits significantly increased between early C₃–C₄ intermediates and the C₄-like intermediates with an operational C₄ cycle. At low CO₂, net photosynthetic rates showed continuous increases from a C₃ state, across the intermediates and towards C₄ photosynthesis, but only C₄-like intermediates and C₄ species (with an operational C₄ cycle) had higher water use efficiencies than C₃ Flaveria. The results demonstrate that both the marginal water use efficiency and the C₄ pump strength increase in C₄ Flaveria to improve their photosynthesis and water use efficiency compared with C₃ species. These findings emphasize that the advantage of the early intermediate stages is predominantly carbon based, not water related.

Key words: C₃–C₄ intermediates, C₃ photosynthesis, leaf gas exchange, photosynthetic model, stomatal conductance, water use efficiency.

Introduction

While only 3% of the world’s terrestrial plant species use the C₄ photosynthetic pathway, C₄ species are responsible for some 20% of global gross primary productivity (Sage et al., 2012). The high productivity of C₄ plants is due to their efficient photosynthetic physiology, which includes an additional yet spatially separated metabolic cycle, mediated by phosphoenolpyruvate carboxylase (PEPCase), to the conventional C₃ Calvin–Benson cycle. This additional cycle results
in high CO₂ concentrations around Rubisco, thus suppressing the enzyme’s oxygenase function and nearly eliminating photorespiration and the associated carbon and energetic costs.

C₄ photosynthesis has evolved independently at least 66 times in lineages throughout the plant kingdom and, in some of these lines, there are intermediate species that are neither C₃ nor fully C₄ (Sage et al., 2012). Phylogenetic analyses in some evolutionary groups where the full range of C₃, C₃–C₄ intermediates, and C₄ species are found (such as the genus Flaveria) have confirmed that C₃ photosynthesis is the basal state and C₄ photosynthesis is more derived; these analyses also place photosynthetic intermediate species as evolutionary intermediates to these two photosynthetic types (McKown et al., 2005). The C₃–C₄ intermediates can be classified into three categories based on the degree to which they express C₄ traits: Type I intermediates show re fixation of photorespiratory CO₂ by Rubisco in enlarged bundle sheath cells; Type II species have increased PEPCase activity and some C₄ cycle function; and C₄-like species have an operational C₄ cycle, but have some residual Rubisco activity in their mesophyll cells (Edwards and Ku, 1987).

Despite extensive research, the role of environmental factors in driving the evolution of C₄ photosynthetic traits continues to draw significant attention (e.g. Osborne and Sack, 2012; Griffiths et al., 2013). Much work has focused on the importance of a drop in atmospheric CO₂ concentrations (eₙ) from near 1000 ppm to ~400 ppm ~30 million years ago (mya), where the predominant benefit from a CO₂-concentrating mechanism would have been enhanced net CO₂ fixation rates through suppression of photorespiration (Ehleringer et al., 1997; Sage, 2004; Christin et al., 2011; Gowik et al., 2011; Sage et al., 2012). While low eₙ increases photorespiration, the effect is even greater when combined with high temperatures: very low eₙ conditions, such as those of the last glacial period (~180 μmol mol⁻¹) (Lüthi et al., 2008), may have selected for traits in some C₃ species to favour the capture and reassimilation of respired and photorespired CO₂ to offset this stress (Busch et al., 2013), but the detrimental effects of low eₙ conditions on C₃ species are further exacerbated at warmer temperatures (Campbell et al., 2005). The warm regions where C₄ species evolved therefore probably stimulated photorespiration considerably, but they also drove a concomitant increase in transpiration demand (Taylor et al., 2012). It has been known for decades that C₄ plants are more water use efficient than C₃ species under the same conditions (e.g. Rawson et al., 1977; Morison and Gifford, 1983); a spate of recent work has highlighted the role of other environmental variables that, along with low eₙ, may have contributed to the rise of C₄ photosynthesis, such as dry or saline conditions. These recent studies have emphasized the role of C₄ photosynthesis in improving plant water status and preventing hydraulic failure in these environments (Osborne and Sack, 2012; Griffiths et al., 2013).

Since C₄ species can maintain high photosynthetic rates even when stomatal conductance is low compared with their C₃ counterparts, it follows that C₄ photosynthesis promotes higher water use efficiencies (WUEs) than are found in C₃ species (e.g. Rawson et al., 1977; Morison and Gifford, 1983; Monson, 1989; Huxman and Monson, 2003; Kocacin ar et al., 2008; Osborne and Sack, 2012). This pattern is apparent in both forms of WUE: instantaneous WUE (WUEᵢ, defined as the leaf carbon gain from net photosynthesis, Aₙₑᵗ, per unit water lost via transpiration, E) and marginal WUE (λ = ∂Aₙₑᵗ / ∂E; see Lloyd and Farquhar (1994); Vogan and Sage (2011); Manzoni et al. (2011); note that this definition of λ is consistent with that of Hari et al. (1986), but the inverse of the same symbol used by Cowan and Farquhar (1977)). The marginal WUE λ can also be interpreted as the cost of losing water in carbon units. Thus, the higher λ of C₄ species implies that water loss is more costly for the carbon balance with respect to C₃ species, so that C₄ species operate at a relatively low E, but at comparable or higher Aₙₑᵗ. This finding is consistent with C₄ photosynthesis preventing hydraulic failure by means of a tight stomatal regulation of water loss (Osborne and Sack, 2012). Yet what controls the stomatal behaviour and WUE across the evolutionary continuum of C₃ to C₄ species remains a subject of research (Vogan and Sage, 2011; Way, 2012) and frames the scope of this work.

Recent experiments have shown that instead of a gradual improvement in WUE from C₃ species, across the intermediate range and to a full C₄ pathway, the increase in WUE resembles a threshold effect: Type I and II intermediates have WUEs on a par with C₃ species, while C₄-like species have a high WUE akin to C₄ plants (Kocacin ar et al., 2008; Vogan and Sage, 2011). The development of the CO₂-concentrating mechanism, which effectively pumps CO₂ from the substomatal cavity into the chloroplasts where the Calvin–Benson cycle occurs, is thought to be the primary mechanism by which C₄ plants enhance their WUE. The present study therefore sought to investigate the connection between the evolutionary continuum of C₃ to C₄ photosynthesis and stomatal behaviour (which is a key factor in controlling WUE), by exploring the following questions.

(i) To what degree can stomatal optimization theories describe the WUE patterns in species that have photosynthetic characteristics intermediate between C₃ and C₄ species?
(ii) What are the relationships among the CO₂-concentrating mechanism, λ, and WUE in these C₃–C₄ intermediates?

To address these questions, the genus Flaveria was used as a case study, since it contains species with C₃ photosynthesis, all three intermediate photosynthetic types, and C₄ photosynthesis (a system previously used by Huxman and Monson, 2003; Sage, 2004; McKown and Dengler, 2007; Kocacin ar et al., 2008; Gowik and Westhoff, 2011; Gowik et al., 2011; Vogan and Sage, 2011; and others). Data on Flaveria are used to parameterize a stomatal optimization model and examine stomatal behaviour across the evolutionary range of C₃ to C₄ photosynthesis. By using a phylogenetically constrained system, the patterns of changes in the model parameters across the C₃–C₄ photosynthetic continuum can be simultaneously explored while minimizing evolutionary differences between groups that might otherwise confound the analysis.
Theory

In the Farquhar et al. (1980) photosynthesis model, \( A_{\text{net}} \) is determined by the minimum of two limitations: the Rubisco carboxylation rate \( (A_C) \) and ribulose-1,5-bisphosphate (RuBP) regeneration rate \( A_I \), and is commonly expressed as

\[
A_{\text{net}} = \min(A_C, A_I) - R_d,
\]

where \( R_d \) is the daytime respiration rate (see Table 1 for symbols and definitions). Rubisco limitation occurs under saturating light or at low CO2 concentrations at the site of Rubisco, while RuBP regeneration tends to limit photosynthesis when \( c_i \) is high and light levels are low, resulting in a limited electron transport rate. The Rubisco-limited assimilation rate, \( A_C \), can be expressed as

\[
A_C = \frac{V_{c,\text{max}} c_i - \Gamma^*}{c_i + K_{c,\text{air}}},
\]

where \( V_{c,\text{max}} \) is the maximum Rubisco carboxylation rate, \( c_i \) is the CO2 concentration at the photosynthetic site, \( \Gamma^* \) is the CO2 compensation point in the absence of mitochondrial respiration, and \( K_{c,\text{air}} = K_c (1 + O / K_o) \), with \( K_c \) and \( K_o \) being the Michaelis–Menten constants for Rubisco CO2 fixation and oxygen inhibition, and \( O \) is the oxygen concentration in the air (21%). Conversely, the RuBP-limited assimilation rate is constrained by the rate of electron transport, \( J \), and can be expressed as

\[
A_I = \frac{J}{4} \frac{c_i - \Gamma^*}{c_i + 2\Gamma^*},
\]

where the electron transport rate is given by \( J = \alpha_\text{p} \epsilon_m Q \), and \( Q \) is the irradiance, \( \alpha_\text{p} \) is the leaf absorptivity, and \( \epsilon_m \) is the maximum photochemical efficiency (Genty et al., 1989).

To avoid discontinuities in \( A_{\text{net}} \) due to an abrupt transition from one limitation to another, the minimum function in Equation 1 has often been replaced by a quadratic function, at the cost of introducing an additional curvature parameter. An alternative approach is to approximate Equation 1 by a hyperbolic function (Vico et al., 2013),

\[
A_{C,J} = k_1 \frac{c_i - \Gamma^*}{c_i + k_2},
\]

where \( k_1 = \frac{J}{4} \) and \( k_2 = JK_{c,\text{air}} / (4V_{c,\text{max}}) \). Such a representation ensures that at low \( c_i / k_2, A_{C,J} = A_C \) and at large \( c_i / k_2, A_{C,J} = A_I \). When \( c_i / k_2 \) is approximately unity, both Rubisco and RuBP regeneration rates exert comparable limitations on photosynthesis. Hereafter, this regime is referred to as the co-limitation regime. Under CO2-limited (or light-saturated) conditions in which \( k_2 = V_{c,\text{max}} \) and \( k_1 = K_{c,\text{air}} \), the optimal solution is identical to the one obtained by Katul et al. (2010) for non-linear photosynthetic kinetics without light limitation. Based on Equation 4, net photosynthesis is obtained as

\[
A_{\text{net}} = A_{C,J} - R_d.
\]

Although there are numerous physiological and anatomical traits that underlie the development of the CO2-concentrating mechanism in C4 plants (e.g. McKown and Dengler, 2007; Sage et al., 2012), for modelling purposes, the simplest description of the effect of such a pump is to assume that the CO2 concentration at the site where photosynthesis occurs is \( c_i = \eta c_o \), where \( \eta \) represent the strength of the CO2-concentrating pump. The value of \( \eta \) encompasses not only the development of C4 biochemistry across the evolutionary gradient of species, but also biochemical and anatomical features that affect mesophyll conductance. In C3 species, \( \eta = 1 \) (Manzoni et al., 2011); while it is slightly smaller than unity in C3 species due to the need to diffuse CO2 through the mesophyll, the lack of specific data on mesophyll conductance meant this had to be neglected and \( \eta = 1 \) was set for C3 species.

In the following, the pump strength, \( \eta \), is estimated from the slope of the \( A_{\text{net}}(c_i) \) curve. Employing this simple description of the CO2-concentrating mechanism results in a simpler photosynthesis model than by considering explicitly PEPCase kinetics (Collatz et al., 1992; Laish and Edwards, 2000; von Caemmerer, 2000, 2013; Vico and Porporato, 2008), thereby allowing data sets collected across different experiments and conditions to be compared. Nevertheless, the parameter \( \eta \) can be linked to the kinetics of PEPCase. The CO2 concentration in the stomatal cavity (\( c_i \)) is assumed to be transported by PEPCase activity and the shuffling of C4 acids to the bundle sheath (the site of photosynthesis), where the CO2 concentration reaches \( c_o \). When PEPCase kinetics are assumed to be linear for illustration (but see von Caemmerer, 2000 for more detailed and non-linear models), then

\[
V_p = \alpha c_i,
\]

where \( \alpha \) is the kinetic constant of the process. Setting \( V_p = A_{\text{net}} \) (from Equation 4) to guarantee continuity in the C fluxes from the stomatal cavity to the site of photosynthesis provides an equation to be solved for \( \eta \), leading to

\[
\eta = \frac{k_1 \Gamma^* + k_2 (\alpha c_o + R_d)}{c_i (k_1 - \alpha c_i - R_d)}.
\]

Equation 6 shows that the pump efficiency \( \eta \) in principle depends on the photosynthetic parameters as well as \( c_i \). However, neglecting \( R_d \) and assuming \( \Gamma^* = < c_i > \) and \( \alpha c_i = < k_1 > \), it can be shown that \( \eta = \alpha k_2 / k_1 \), which is a constant at a given temperature and light level. Therefore, when respiration is small and photosynthetic capacity is large, a constant efficiency \( \eta \) captures the main effect of the PEPCase on the photosynthetic rate. Outside these simplifications, the assumption of a constant \( \eta \) can only be regarded as a first-order approximation.

The combination of the hyperbolic function in Equation 4 with the simplified description of the CO2 pumping mechanism based on \( \eta \) (i.e. \( c_i = \eta c_o \)) provides a tool to describe CO2 demand within a common framework valid across the C3 to C4 evolutionary continuum. Despite the inherent simplifications, this model is in good agreement with earlier, more complex photosynthesis models for C3-C4 intermediates and C4 species (von Caemmerer, 1989; Collatz et al., 1992) (data not shown; for an example of model comparison for C3 species, see fig. 1 in Vico et al., 2013), thus lending support to the present approach.
The biochemical demand for CO₂ described by $A_{CJ}$ is met by CO₂ supplied by the atmosphere via Fickian diffusion at a rate given by

$$A_{\text{net}} = g_s (c_a - c_i),$$  \hspace{1cm} (7)

where $g_s$ is the stomatal conductance and $c_a$ is the atmospheric CO₂ concentration. For a given $c_a$, set of environmental conditions (such as $Q$ and temperature), and physiological properties determining $k_1$ and $k_2$, the atmospheric supply and biochemical demand for $A_{CJ}$ constitute two equations with three unknowns: $g_s$, $c_a$, and $A_{\text{net}}$. Hence, one additional equation is needed to close this system of equations mathematically.

This additional equation can take on the form of an optimality rule, whereby stomata are assumed to operate so as to maximize their carbon gain at a given water loss cost (Cowan and Farquhar, 1977; Hari et al., 1986). This hypothesis is equivalent to maximizing a Hamiltonian function $H=A_{\text{net}} - \lambda E$, where $E=ag_sD$ is the leaf transpiration rate (assuming a perfectly coupled canopy), and $a=1.6$ is the ratio of the molecular diffusivities of CO₂ to water vapour. Combining the biochemical demand with atmospheric supply so as to eliminate $c_i$, and thereby expressing $A_{CJ}$ as a function of $g_s$, inserting the outcome into the Hamiltonian, and setting $\partial H / \partial g_s = 0$, leads to a quadratic equation in $g_s$ (Vico et al., 2013). Solving this equation for $g_s$ results in a solution for optimal $g_s$ as a function of biochemical parameters ($\eta$, $V_{c,max}$, $K_{\text{air}}$, $R_0$, and $\Gamma^*$), environmental conditions ($c_a$ and $D$), and the optimization parameter $\lambda$. The explicit functional form for optimal stomatal conductance is determined from the solution to the optimality problem as

### Table 1. Symbols and their definitions used in the paper and model

| Symbol | Definition | Units |
|--------|------------|-------|
| $a$   | Ratio of the molecular diffusivities of CO₂ to water vapour | – |
| $A_C$ | Rubisco-limited CO₂ assimilation rate | $\mu$mol m$^{-2}$ s$^{-1}$ |
| $A_J$ | RuBP regeneration-limited net CO₂ assimilation rate | $\mu$mol m$^{-2}$ s$^{-1}$ |
| $A_{\text{net}}$ | Net CO₂ assimilation rate | $\mu$mol m$^{-2}$ s$^{-1}$ |
| $c_a$ | Atmospheric CO₂ concentration | $\mu$mol mol$^{-1}$ |
| $c_{bs}$ | Bundle sheath CO₂ concentration | $\mu$mol mol$^{-1}$ |
| $c_c$ | Chloroplastic CO₂ concentration | $\mu$mol mol$^{-1}$ |
| CE | Carboxylation efficiency | – |
| $c_i$ | Intercellular CO₂ concentration | $\mu$mol mol$^{-1}$ |
| $D$ | Vapour pressure deficit | kPa |
| $E$ | Transpiration rate | mmol m$^{-2}$ s$^{-1}$ |
| $g_s$ | Stomatal conductance | mmol m$^{-2}$ s$^{-1}$ |
| $J$ | Electron transport rate | – |
| $k_1$ | Maximum photosynthetic rate of the hyperbolic model (Equation 4) | $\mu$mol m$^{-2}$ s$^{-1}$ |
| $k_2$ | Half-saturation constant for the hyperbolic model (Equation 4) | $\mu$mol m$^{-2}$ s$^{-1}$ |
| $K_c$ | Michaelis–Menten constant for Rubisco carboxylation | $\mu$mol mol$^{-1}$ |
| $K_{\text{air}}$ | Half-saturated constant of the Rubisco-limited photosynthesis | mmol mol$^{-1}$ |
| $K_0$ | Michaelis–Menten constant for Rubisco oxygenation | mmol mol$^{-1}$ |
| $O$ | Atmospheric oxygen concentration | – |
| $Q$ | Irradiance | WUE | Instantaneous water use efficiency | mmol mol$^{-1}$ |
| $V_{c,\text{max}}$ | Maximum carboxylation rate of Rubisco | $\mu$mol m$^{-2}$ s$^{-1}$ |
| $V_{c,\text{max}25}$ | Maximum carboxylation rate of Rubisco at 25°C | $\mu$mol m$^{-2}$ s$^{-1}$ |
| $V_p$ | PEPCase rate | $\mu$mol m$^{-2}$ s$^{-1}$ |
| WUE | Water use efficiency | mmol mol$^{-1}$ |
| $\alpha$ | Kinetic constant (Equations 5 and 6) | – |
| $\alpha_{p}$ | Leaf absorptivity | – |
| $\alpha_{1,2}$ | Parameter groups (Equations 8–11) | – |
| $\beta_{1,2,3}$ | Parameter groups (Equations 8–11) | – |
| $\epsilon_m$ | Maximum photochemical efficiency | – |
| $\gamma$ | Parameter groups (Equations 8–11) | – |
| $\Gamma$ | CO₂ compensation point | $\mu$mol mol$^{-1}$ |
| $\Gamma^*$ | CO₂ compensation point in the absence of mitochondrial respiration | $\mu$mol mol$^{-1}$ |
| $\eta$ | C4 pump strength | – |
| $\lambda$ | Marginal water use efficiency | mmol mol$^{-1}$ |

*By guest on 30 July 2018*
\[
\frac{\partial H}{\partial g_s} = \frac{\partial (A_{net} - \lambda E)}{\partial g_s} = 0 \quad \text{optimality condition}
\]
\[
g_s = \frac{\beta_1 + \sqrt{\beta_2}}{\beta_3}
\]  
\(8\)

where

\[
\beta_1 = -\gamma \eta (k_2 + \alpha_2) \left[ k_1 (k_2 - \eta c_a + 2\Gamma^*) + \alpha_i R_d \right],
\]
\(9\)

\[
\beta_2 = -\gamma k_1 (k_2 + \Gamma^*) (k_2 + \alpha_2)
\]
\[
\left[ k_1 + \eta (c_s - 2\gamma) \right]^2 \left[ k_1 (\Gamma^* - \eta c_s) + \alpha_i R_d \right],
\]
\(10\)

\[
\beta_3 = \gamma c_s^2 (k_2 + \alpha_2)
\]
\(11\)

In Equations 9, 10, and 11, \(\alpha_i = \eta k_s + \alpha_s, \alpha_s = \eta (c_s - \gamma), \) and \(\gamma = \omega D.\) Therefore, the optimal stomatal conductance depends on \(\lambda,\) which by using the optimization condition \(\partial H / \partial g_s,=0\) can be shown to be equal to the definition of the marginal WUE, i.e.

\[
\lambda = \frac{\partial A_{net}}{\partial E} = \frac{\partial A_{net}}{\partial g_s} \left( \frac{\partial E}{\partial g_s} \right)^{-1}.
\]
\(12\)

Equation 12 provides a physical interpretation for \(\lambda,\) but does not give additional information (the optimization condition has already been used in Equation 8). Hence, \(\lambda\) needs to be determined to close the optimization problem mathematically. Although \(\lambda\) changes as a function of time when soil moisture declines during a dry period (Manzoni et al., 2013), under well-watered conditions or stable moisture levels, \(\lambda\) can be considered time-invariant.

Before applying the proposed model, it is important to summarize its key assumptions and simplifications:

(i) Photosynthetic kinetics are described by a hyperbolic function of \(c_1,\) bridging a CO\(_2\)-limited regime (where \(A_{net}\) scales linearly with \(c_1\)) and a light-limited regime (where \(A_{net}\) depends solely on a light level).

(ii) PEPCase kinetics are described by a single efficiency parameter \(\eta,\) which approximates more complex models well (Collatz et al., 1992; von Caemmerer, 2000) when respiration terms are small. Also mesophyll resistance is neglected, due to a lack of data across these species; this assumption implies that the estimated \(\eta\) could be inflated under dry conditions (though these are not the conditions considered when inferring the marginal WUE).

(iii) Stomatal conductance is obtained from an optimization argument assuming that the marginal WUE is constant—a reasonable approximation for experiments under controlled conditions and stable moisture levels (Manzoni et al., 2013). Thus, \(\lambda\) is used as a fitting parameter affecting the stomatal conductance in Equation 8.

Clearly, these assumptions could be relaxed, thereby improving realism. However, relaxing these assumptions reduces the ease of interpretation of the derived equations and the ability to compare across a wider range of data sets due to more required parameters. Once the optimal \(g_s,\) is determined, \(A_{CJ}, E,\) and \(c_1\) can then be computed. This model allows the quantification of \(A_{net}\) and \(g_s\) for \(C_3,\) \(C_4,\) and \(C_3-C_4\) intermediate species within a common framework and as a function of both environmental conditions (air temperature, \(Q, D,\) and \(c_4\)) and species-specific parameters (\(\eta, \lambda, V_{c,max}, K_{air}\), and \(\Gamma^*\)). As such, after showing that the modelled response of \(A_{net}\) to changes in \(g_s\) is well captured assuming optimal stomatal behaviour, the model is used to investigate how \(A_{net}\) and WUE, are altered by changes in \(\eta, \lambda,\) and \(c_4;\) thus following the steps of the hypothesized evolution of \(C_3-C_4\) intermediates and \(C_4\) species from \(C_3\) plants.

Data availability and model parameterization

To examine the consequences of intermediacy on stomatal behaviour, WUE\(_i,\) and \(\lambda,\) \textit{Flaveria} species were used where gas exchange measurements for \(C_3,\) \(C_4,\) and intermediate species have been previously characterized. The data included \(C_3\) species (\(F.\) \textit{cronquistii} and \(F.\) \textit{pringleii}), Type I intermediates (\(F.\) \textit{angustifolia}, \(F.\) \textit{chloraeofolia}, \(F.\) \textit{pubescens} and \(F.\) \textit{sonoren sis}), Type II intermediates (\(F.\) \textit{floridana} and \(F.\) \textit{ramossissima}), \(C_4\)-like intermediates (\(F.\) \textit{browni}, \(F.\) \textit{palmeri} and \(F.\) \textit{vagnata}), and \(C_4\) species (\(F.\) \textit{australisca}, \(F.\) \textit{bidentis}, \(F.\) \textit{kochiana} and \(F.\) \textit{trinervia}) (see Supplementary Table S1 available at JXB online). The most recent photosynthetic classification of the species (Vogan and Sage, 2011) was employed. Responses of \(g_s\) and \(A_{net}\) to variation in \(c_a\) and responses of \(A_{net}\) to changes in \(g_s\) were either taken from the literature or digitized from published graphs (Monson, 1989; Vogan and Sage, 2011). Environmental conditions (\(Q, D, c_o,\) and leaf temperature) in model runs were matched to the measurement conditions described for the experimental data. The range in \(\lambda\) necessary to capture measured responses in gas exchange was explored in \textit{Flaveria} species from all photosynthetic types.

To parameterize the above model for \textit{Flaveria}, \(V_{c,max}\) values were derived for Rubisco from 15 \textit{Flaveria} species that spanned \(C_3\) to \(C_4\) photosynthetic types using \textit{in vitro} measurements of catalytic constants (or turnover numbers, \(k_{cat}\)) (Kubien et al., 2008) and Rubisco site concentrations from the same experiment (D. Kubien, personal communication) (Table 2). The Michaelis–Menten constants \(K_c\) and \(K_o\) for Rubisco were also taken for each \textit{Flaveria} species from Kubien et al (2008). Rubisco kinetics were adjusted to 30 °C to match conditions in the carboxylation efficiency studies using correction equations and coefficients from Campbell and Norman (1998) (Table 2). \(K_c\) and \(K_o\) were temperature adjusted by multiplying their values at 25 °C by \(exp[\{q(T_l-25)\}],\) where \(q\) is the temperature coefficient for that parameter (0.074 for \(K_c\) and 0.015 for \(K_o\)) and \(T_l\) is leaf temperature. \(V_{c,max}\) was adjusted as \(V_{c,max} = V_{c,max25} \times exp(0.088(T_l-25)) [1+exp(0.29(T_l-41))],\) where \(V_{c,max25}\) is the maximum carboxylation rate at 25 °C. The \(\Gamma^*\) values for each of the five photosynthetic types were approximated using averaged values of \(\Gamma\) (the CO\(_2\) compensation point) from \textit{Flaveria} species in Ku et al. (1991), assuming that day respiration of mitochondria is small (\(R_d=0.015V_{c,max}\)) and can be ignored (Table 2). Because light conditions varied across experiments, the estimation of \(J(\alpha_s e_m Q)\) needed
Table 2. Parameter values (based on mean values from experimental data corrected to 30 °C) used for modelling photosynthesis for each photosynthetic type of Flaveria

| References for values | CE (mol m⁻² s⁻¹) | Γ (µmol mol⁻¹) | V_{c,max} (µmol m⁻² s⁻¹) | K_c (µmol mol⁻¹) | K_i (mmol m⁻² s⁻¹) | R_d (µmol m⁻² s⁻¹) | λ (mmol mol⁻¹) | η (unitless) |
|-----------------------|------------------|----------------|--------------------------|-----------------|-------------------|-----------------|----------------|-------------|
| Sudderth et al. (2007), citing Dai et al. (1996) | C_3 species | 0.11 | 61.36 | 53.20 | 494.9 | 575.8 | 0.8 | 0.826 | 1.83 |
| Ku et al. (1991) | Type I | 0.079 | 25.45 | 73.40 | 516.6 | 665.7 | 1.10 | 0.739 | 1.73 |
| Kubien et al. (2008) (and pers. comm.) | Type II | 0.13 | 9.20 | 66.72 | 547.1 | 614.6 | 1.00 | 0.754 | 1.93 |
| Kubien et al. (2008) | C_4-like | 0.27 | 4.93 | 34.10 | 690.3 | 1631.8 | 0.59 | 3.410 | 17.60 |
| Estimated as 0.015 V_{c,max} | C_4 species | 0.47 | 3.32 | 39.52 | 898.7 | 1631.8 | 0.59 | 3.410 | 17.60 |

CE, carboxylation efficiency; Γ, CO₂ compensation point; V_{c,max} in vitro maximum carboxylation rate of Rubisco; K_c and K_i (at 30 °C), Rubisco Michaelis–Menten constants for CO₂ and O₂, respectively; R_d, day respiration rates; λ, marginal water use efficiency; η, C_4 pump strength.

Data are taken from literature sources as outlined in the text (see also species-specific data points in Fig. 2 and Supplementary Table S1 at JXB online); λ and η are calculated values.

in determining \( \alpha_c = J_2 = J K_c / (4 V_{c,max}) \) requires an estimate of the product \( \alpha_c \varepsilon_m \) (not their individual values). The value for \( \alpha_p \) had set at 0.8 (based on values for C_3 and C_4 species in von Caemmerer, 2000 and Collatz et al., 1992, respectively). \( \varepsilon_m \) was 0.1 mol mol⁻¹ (similar to Norman and Campbell, 1998; Cheng et al., 2001; Taiz and Zeiger, 2010), and \( Q \) was set for the irradiance used in individual papers being modelled. Hence, \( \alpha_p = 0.8 \lambda \) when \( c_i = 0.08 \lambda \) or \( c_i = 0.08 \lambda \) (assuming \( c_i = 0.08 \lambda \)).

Formal optimization model was simplified by selecting light-saturated \( V_{c,max} \) (assuming \( c_i = 0.08 \lambda \)).

The value for \( \alpha_p \) was set at 0.8 (based on values for C_3 and C_4 species (Campbell and Norman, 1998; see table 14.1) though \( \alpha_p \) may be more uncertain for C_4 species and C_3–C4 intermediates. For C_3–C_3 intermediates, Monson (1989) and Monson and Jaeger (1991) report mid-day photosynthetic rates for several species including F. floridana between 15 µmol m⁻² s⁻¹ and 45 µmol m⁻² s⁻¹ at light levels ranging from \( Q = 1500 \mu mol \mu mol^{-1} \) to 2000 µmol m⁻² s⁻¹. Because \( A_j = J / 4 \) (assuming that \( \Gamma \) is negligible in Equation 3), it follows that the measured \( A_j \) is consistent with the estimate \( J = 0.08 \lambda \), which gives an \( A_{net} \) of 40 µmol m⁻² s⁻¹. This evidence supports the assumption that \( \alpha_p = \varepsilon_m \) is stable across photosynthetic types. A more rigorous parameterization would require direct observations of \( \alpha_p \) or reliable \( V_{c,max} \) versus \( J_{max} \) relationships across the C_3–C_4 continuum.

Carboxylation efficiencies [CEs; i.e. the initial slope of the \( A_{net}(c_i) \) curve] measured under saturating light for Flaveria species were taken from Klarr et al. (1991) and Sudderth et al. (2007), citing Dai et al. (1996). Based on Equation 2, at low \( c_i \) the slope of the \( A_{net}(c_i) \) is approximately \( CE = V_{c,max} \eta / K_{car} \), so that \( \eta = CE / K_{car} \).

Therefore, knowledge of \( CE, K_{car}, K_c, \) and \( K_i \) allowed an estimate of \( \eta \) for each Flaveria species.

Finally, the Vogan and Sage (2011) gas exchange data set was used to infer how \( \lambda \) changes across the evolutionary pathway from C_3 to C_4 species. In that study, a range of \( g_s \) and \( A_{net} \) values was obtained by altering the nitrogen availability for individuals of all photosynthetic types considered, while water was amply supplied, so \( \lambda \) can be considered time-invariant. As a consequence of different nutrient availability, a range of photosynthetic capacities and respiration rates were obtained. Since there is no way of knowing these biochemical parameters, a simplified but more robust approach to estimate \( \lambda \) was adopted that only requires gas exchange rates and photosynthetic type-averaged \( \Gamma \) and \( \eta \) (assuming \( \lambda \) is substantially unaltered by nutrient availability). For this step, instead of using the definition (Equation 12), which requires knowledge of all the photosynthetic parameters, the stoma-atal optimization model was simplified by selecting light-saturated conditions, so that \( R_d = 0 \) and the photosynthesis model is approximately linear. Following these simplifications, it can be shown that \( A_{net} = g_s \sqrt{\alpha p / \eta} \) (Manzoni et al., 2011), which allows estimating \( \lambda \) through a linear least square regression of \( A_{net} \) versus \( g_s \), constrained through the origin for each photosynthetic type. In previous works on different species, this approach to estimate \( \lambda \) was compared with results obtained without these simplifications. Such comparison showed that the differences between the two approaches was rarely more than 20% across a wide range of environmental and physiological conditions (see fig. 4 in Katul et al., 2010), which is in the range of experimental variability [e.g. a mean standard deviation of 16% in light-saturated \( A_{net} \) estimates across individuals in a range of C_3, C_3–C_4 intermediates, and C_4 species (Vogan et al., 2007)].

Gas exchange rates were also simulated under altered atmospheric CO₂ concentrations. In this analysis, all biochemical parameters were maintained constant, but the possibility was considered that the marginal WUE increases linearly with CO₂ concentrations (Manzoni et al., 2011). Simulations with constant \( \lambda \) estimated as described above were thus compared with simulations with \( \lambda(c_i) = \lambda(c_i) / 400 \). Including CO₂ effects allows the robustness of the results to changes in \( \lambda \) to be tested.

Results and Discussion

Recent work has stimulated new interest in the role that transpiration demands may have played in the evolution of C_4 photosynthesis and traits associated with the C_4 syndrome (Taylor et al., 2011, 2012; Osborne and Sack, 2012; Griffiths...
et al., 2013). These studies have emphasized that C₄ photosynthesis not only benefits the carbon economy of a plant, but also has important implications for hydraulic traits, drought tolerance, and water use patterns, benefits that are maintained or enhanced when C₄ plants are exposed to the low cₜ conditions where C₄ photosynthesis evolved (Ripley et al., 2013). Here, λ is used as an ‘index’ of the cost of losing water in terms of carbon, and its variation along the evolutionary gradient from C₃ to C₄ photosynthesis is investigated.

Combining a stomatal optimization approach with measured biochemical parameters, realistic mean Aₙ₉ₐ(c) curves for each photosynthetic pathway in Flaveria were computed (Fig. 1A; Table 2). The corresponding estimated η values are reported in Fig. 1B. In the optimality model, recall that the parameter η represents an overall pump strength for the carbon-concentrating mechanism, which might naively have been expected to increase gradually from C₃ towards C₄ plants. Instead, the analysis here suggests that η was relatively stable and similar to that for C₃ species (η=1 or slightly above 1 due to unavoidable errors in the estimation) until reaching C₄-like species. The relatively constant η between C₃, Type I, and Type II Flaveria species occurred despite there being an increase in the initial slope of the Aₙ₉ₐ(c) curve (e.g. the carboxylation efficiency) across these groups. Instead of being attributed to η, the steeper initial slopes in the Type I and Type II intermediates in comparison with the C₃ species were caused by higher Vₖₘₐₓ values for Rubisco based on in vitro assays of the enzyme kinetic parameters (Table 2; Fig. 2A), consistent with positive selection on Rubisco across the C₃ to C₄ gradient (Kapralov et al., 2011). Thus, there was no increase in η until the C₄-like species were reached; at this point, η values were about half-way between those of the full C₃ and C₄ photosynthetic groups. The greater variation in η estimates in species closer to the C₄ end of the spectrum is therefore probably due to the greater range of pump strengths possible as the carbon-concentrating mechanism is established and to the species-level diversity in Vₖₘₐₓ values (Supplementary Table S1 at JXB online). While there were sharp changes in in vitro Rubisco Vₖₘₐₓ between C₃ species and the Type I and Type II intermediates, the change in the K₉ₐ of Rubisco across the photosynthetic groups was more gradual until reaching the C₄-like species (Fig. 2B), implying that these enzyme kinetic traits are not necessarily linked. The η* dropped sharply as η increased slightly above a value of 1 and then flattened (Fig. 2C).

While it might, a priori, seem reasonable to expect a steady increase in WUE from C₃ species through the intermediate Flaveria species and to full C₄ plants, this was not borne out by the data, in agreement with published findings. Huxman and Monson (2003) showed that the WUEₚ of C₃–C₄ intermediates were similar to C₃ WUEₚ values, while C₄ WUEₚ values were considerably higher. Vogan and Sage (2011) also found no evidence for a gradual transition in the slope between Aₙ₉ₐ and gₛ, in C₃–C₄ intermediates, but rather a sharp increase between Type II intermediates and C₄-like intermediates. [Note that WUE is a proxy for λ if a linear Aₙ₉ₐ(c) curve is assumed.] In our re-analysis of the Vogan and Sage (2011) data set, the reported gas exchange data could be readily described with the optimality model for C₃ and Type I and Type II intermediates using the estimated changes in η (as in Fig. 1B), but without significant changes in λ across photosynthetic pathways (Fig. 3). This result implies that there is little change in the relationship between carbon and water from that of a C₃ species in these early intermediate steps. However, in C₄-like intermediates, a strong C₄ pump (i.e. η=8) was accompanied by a quadrupling of λ compared with that used to characterize the data for Type I and II intermediates. Thus, the stomatal optimization approach could be successfully used to capture key changes in the measured relationship between Aₙ₉ₐ and gₛ across the C₃–C₄ spectrum using the estimated η values, but only when the marginal WUE of the C₄-like and C₄ species was modelled to be 4-fold greater than that of the C₃ species (Fig. 3). This increase in λ unambiguously indicates a higher carbon cost for losing water in the C₄-like and C₄ species. In the optimization model, the long-term cₛ/cₚ dictates λ, because λ∞(1−cₛ/cₚ)². Therefore, the increase in λ is generated by a decline in cₛ (where cₛ is assumed to be 400 μmol mol⁻¹). This finding suggests that the increase in C₄ WUE values, the increase in λ, and the decline in cₛ (as expected by the presence of a C₄ pump) are all interconnected and predicted from the proposed stomatal optimization model.

Fig. 1. (A) Responses of net CO₂ assimilation rates (Aₙ₉ₐ) to increases in intercellular CO₂ concentration (cₚ), relativized to maximum Aₙ₉ₐ (Aₙ₉ₐₘₚ) for each photosynthetic type, as commonly presented in the literature. (B) Estimated η for each photosynthetic type. Means ±SE, n indicated at the top, dotted line indicates η=1. C₃ species, purple circles and solid line; Type I species, blue diamonds and dashed line; Type II species, green triangles and dotted line; C₄-like species, yellow inverted triangles and dashed-dotted line; C₄ species, red squares and solid line.
WUE$_i$ was further investigated under both current (400 µmol mol$^{-1}$) and low CO$_2$ concentrations (280 µmol mol$^{-1}$; Figs 4, 5), allowing $V_{c,max}$, $K_{cair}$, and $\Gamma^*$ to vary along with $\eta$ as per the relationships in Figs 1 and 2, but keeping $\lambda$ constant. In the results from both current and low $c_a$ levels, increases in $\eta$ initially induce a sharp increase in $A_{net}$, as more CO$_2$ is concentrated around Rubisco, with a diminishing response above a certain $\eta$ ($\eta=10$ for 400 µmol mol$^{-1}$ CO$_2$; Fig. 4A). Moreover, higher values of $\lambda$ decrease $A_{net}$ in both environments, so that the slight changes in $\eta$ and $\lambda$ in Type I and II species compared with C$_3$ plants generate a similar $A_{net}$ in the three groups (Fig. 4A). Increased $c_a$ (from 280 µmol m$^{-2}$ s$^{-1}$ to 400 µmol mol$^{-1}$) elevated $A_{net}$ in C$_3$ species by 80% due to greater substrate availability (Fig. 4B). Compared with a C$_3$ Flaveria at these low $c_a$, C$_3$–C$_4$ intermediates also have higher $A_{net}$ in modern CO$_2$ concentrations, with a gradual increase in the stimulation of $A_{net}$ with respect to C$_3$ values (Fig. 4B). In contrast to the $A_{net}$ results, increases in $\eta$ have little impact on WUE$_i$ when $\lambda$ is small, namely from C$_3$ species to Type I or II species (shown for 400 µmol mol$^{-1}$ CO$_2$ in Fig. 5A). Moreover, while increases in $c_a$ have increased WUE$_i$ of C$_3$ species by 30%, there is no gradual rise in WUE$_i$ across the gradient of photosynthetic types, as there was with $A_{net}$ (Fig. 5B). Instead, compared with a C$_3$ Flaveria at 280 µmol mol$^{-1}$ CO$_2$, Type I and Type II intermediates have a similar 30% stimulation in WUE$_i$, while C$_4$-like and C$_4$ species show a more than tripling of their WUE$_i$ stimulation at modern CO$_2$ levels (Fig. 5B).

If the marginal WUE is assumed to increase with atmospheric CO$_2$ (e.g. Katul et al., 2010; Manzoni et al., 2011), the predicted $g_i$ at $c_a=280$ µmol mol$^{-1}$ increases. As a consequence, photosynthesis also increases and the ratios of net photosynthesis at $c_a=400$ µmol m$^{-2}$ s$^{-1}$ and 280 µmol mol$^{-1}$ therefore decrease (Fig. 4B). Because the positive effect of changes in $\lambda$ is larger on transpiration than on net photosynthesis, the WUE$_i$ at the lower $c_a$ decreases. As a result, the ratio of WUE$_i$ at current and low CO$_2$ concentrations is higher than when assuming a constant $\lambda$ (Fig. 5B).

The modelled changes in leaf-level performance between photosynthetic groups under low $c_a$ are shown in Fig. 6. This figure quantifies the advantages of the intermediate and C$_4$ species over the basal C$_3$ state. At low $c_a$, the estimated changes in $\eta$ and $\lambda$ for intermediate species provide a continuous, smooth gradient of increasing carbon gain, over a C$_3$ Flaveria species (Fig. 6A). This trend is robust to changes in the $\lambda(c_a)$ relationship, as indicated by minor differences between filled and open symbols. A Type I intermediate has a 13% higher $A_{net}$ than a C$_3$ species, which could provide a competitive edge to the intermediate in a low CO$_2$ environment; a similar jump in $A_{net}$ is seen for each photosynthetic group along the evolutionary trajectory, in agreement with a recently proposed smoothly increasing fitness landscape for C$_4$ evolution (Heckmann et al., 2013). However, the same pattern is not apparent in the WUE$_i$ results (Fig. 6B). There is no difference in the WUE$_i$ estimated at 280 µmol mol$^{-1}$ CO$_2$ between C$_3$, Type I and Type II Flaveria species. Instead, significant increases in WUE$_i$ are only achieved in C$_4$-like and C$_4$ species, implying that the driving force for the initial steps.
Fig. 3. Relationships between stomatal conductance to CO₂ ($g_s$) and net CO₂ assimilation rate ($A_{\text{net}}$) in Flaveria species across the C₃ to C₄ photosynthetic range. Data points are from Vogan and Sage (2011); lines are obtained by analytical least-square fitting of the water use efficiency $\lambda$, employing a linearized version of the stomatal optimization model (Manzoni et al., 2011) for analytical tractability.

Fig. 4. (A) Modelled relationships between net CO₂ assimilation rate ($A_{\text{net}}$), marginal water use efficiency ($\lambda$), and the CO₂-concentrating pump strength ($\eta$) modelled at current CO₂ concentrations (400 μmol mol⁻¹); $V_{c,\text{max}}$, $K_{\text{car}}$, and $\Gamma^*$ vary with $\eta$ according to the relationships in Fig. 2; vapour pressure deficit ($D$) was set to 1.5 kPa, leaf temperature to 30 °C, $Q$ to 1500 μmol m⁻² s⁻¹. Mean values of $\lambda$ and $\eta$ for each of the five photosynthetic types are indicated on the surface. (B) The ratio of $A_{\text{net}}$ at current atmospheric CO₂ levels versus $A_{\text{net}}$ of C₃ Flaveria at low atmospheric CO₂ concentrations (280 μmol mol⁻¹) ($A_{\text{net},400}/A_{\text{net},280}$) for each photosynthetic group; means ±SE across species; filled symbols refer to constant $\lambda$, open symbols to $\lambda$ increasing linearly with $c_a$; the dashed-dotted line indicates a ratio of 1. C₃ species, purple circle; Type I species, blue diamond; Type II species, green triangle; C₄-like species, yellow inverted triangle; C₄ species, red square.

Fig. 5. Modelled relationships between instantaneous water use efficiency (WUE, the ratio of $A_{\text{net}}$ to $E$), marginal WUE ($\lambda$), and the CO₂-concentrating pump strength ($\eta$) modelled at current CO₂ concentrations (400 μmol mol⁻¹); $V_{c,\text{max}}$, $K_{\text{car}}$, and $\Gamma^*$ vary with $\eta$ according to the relationships in Fig. 2; vapour pressure deficit ($D$) was set to 1.5 kPa, leaf temperature to 30 °C, $Q$ to 1500 μmol m⁻² s⁻¹. Mean values of $\lambda$ and $\eta$ for each of the five photosynthetic types are indicated on the surface. (B) The ratio of WUE at current atmospheric CO₂ levels versus the WUE of a C₃ Flaveria at low atmospheric CO₂ concentrations (280 μmol mol⁻¹) (WUE₄₀₀/WUE₂₈₀) for each photosynthetic group; means ±SE across species; filled symbols refer to constant $\lambda$, open symbols to $\lambda$ increasing linearly with $c_a$; the dashed-dotted line indicates a ratio of 1. C₃ species, purple circle; Type I species, blue diamond; Type II species, green triangle; C₄-like species, yellow inverted triangle; C₄ species, red square.
from C3 to C4 photosynthesis, with C4 species having lower WUEi among Flaveria species with different photosynthetic types at $c_3=280$ μmol mol$^{-1}$, expressed as ratios over the mean $A_{net}$ and WUE for C3 species at $c_3=280$ μmol mol$^{-1}$. Symbols represent means ±SE across species (for fixed $c_3$, $A_{net}$ and WUEs values); filled symbols refer to constant $\lambda$, open symbols to $\lambda$ increasing linearly with $c_3$; the dashed-dotted line indicates a ratio of 1. Other parameters are as in Figs 4 and 5.

Many of the features considered to pre-adapt a group to evolve C4 photosynthesis are related to leaf hydraulics, including increased vein density and enlarged bundle sheath cell size (McKown et al., 2005; McKown and Dengler, 2007; Osborne and Sack, 2012; Sage et al., 2012; Griffiths et al., 2013). Stomatal anatomy also evolves along the transition from C3 to C4 photosynthesis, with C4 species having lower maximum stomatal conductance (due to either lower stomatal density or smaller stomatal size) than C3 congeners (Taylor et al., 2012). While changes in whole-plant physiology are outside the scope of this work, these findings have stimulated interest in the role of plant water relations in the evolution of C4 photosynthesis. The results here indicate that while there is a gradual increase in carbon gain across the range from C3 to C4, there is no corresponding transition in either WUEi or $\lambda$. Rather, increases in leaf-level WUEi are only seen between Type II intermediacy and C4-like species (as noted by Kocacinar et al., 2008; Vogan and Sage, 2011). However, this transition is accompanied by a rise in $\lambda$, indicating that a higher carbon cost is being incurred for water loss in C4-like and C4 species than in C3, or Type I or Type II intermediate species of Flaveria. This corresponds to the coordinated set of changes to the hydraulic architecture of Flaveria species, including lower leaf specific hydraulic conductivity and greater cavitation resistance in C4 and C4-like than C3 species (Kocacinar et al., 2008), emphasizing the importance of the transition from having a functional C4 cycle for both the carbon and water economies of the plant.

**Conclusions**

Using a stomatal optimization approach, the full range of C3, C3–C4 intermediates, and C4 gas exchange could be realistically modelled with the addition of a C4 pump strength parameter $\eta$, describing the effects of the C4 carbon-concentrating mechanism. The results here showed that, to capture the patterns apparent in measured gas exchange data, the carbon-based cost of losing water ($\lambda$) between C3, and Type I and Type II intermediates could be maintained constant, but $\lambda$ had to be quadrupled to model C4-like and C4 Flaveria (at least within the confines of the optimality assumption of stomata). When leaf-level fluxes were modelled at low CO2, there was no evidence for a greater WUEi in the C3–C4 intermediates (compared with a C4 Flaveria) until they developed a full C4 cycle. However, the model results suggest a steady increase in net carbon fixation rates across the C3 to C4 photosynthetic range. While this implies that carbon, not water, was the main driving pressure for the early steps of C4 evolution in this genus, the increase in $\lambda$ indicates that there was a fundamental shift over the evolution of C4 photosynthesis between the relative costs of carbon and water, resulting in higher carbon costs of water losses.

**Supplementary data**

Supplementary data are available at *JXB* online.

**Table S1. Parameter table.**

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**References**

Busch FA, Sage TL, Cousins AB, Sage RF. 2013. C3 plants enhance rates of photosynthesis by reassimilating respired CO2. *Plant, Cell and Environment* 36, 200–212.

Campbell GS, Norman JM. 1998. An introduction to environmental biophysics. 2nd edn. Berlin: Springer.

Campbell CD, Sage RF, Kocacinar F, Way DA. 2005. Estimation of the whole-plant CO2 compensation point of tobacco (Nicotiana tabacum L.). *Global Change Biology* 11, 1956–1967.
Cheng L, Fuchigami LH, Breen PJ. 2001. The relationship between photosystem II efficiency and quantum yield for CO2 assimilation is not affected by nitrogen content in apple leaves. Journal of Experimental Botany 52, 1865–1872.

Christin PA, Osborne CP, Sage RF, Arakaki M, Edwards EJ. 2011. C4 eudicots are not younger than C3 monocots. Journal of Experimental Botany 62, 3171–3181.

Collatz, GJ, Ribas-Caro M, Berry JA. 1992. Coupled photosynthesis–stomatal conductance model for leaves of C4 plants. Australian Journal of Plant Physiology 19, 519–538.

Cowen RD, Farquhar GD. 1977. Stomatal function in relation to leaf metabolism and environment. Symposia of the Society for Experimental Biology 31, 471–505.

Dai, Z, Ku MSB, Edwards GE. 1996. Oxygen sensitivity of photosynthesis and photorespiration in different photosynthetic types in the genus Flaveria. Planta 186, 563–571.

Edwardes GE, Ku MSB. 1987. Biochemistry of C3–C4 intermediates. In: Stumpf PK, Conn EE, eds. The biochemistry of plants. London: Academic Press, 275–325.

Ehleringer JR, Cerling TE, Helliker BR. 1997. C4 photosynthesis, atmospheric CO2, and climate. Oecologia 112, 285–299.

Farquhar G, von Caemmerer S, Berry JA. 1980. A biochemical model of photosynthetic CO2 assimilation in leaves of C3 species. Planta 149, 78–90.

Genty B, Briantais JM, Baker NR. 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochimica et Biophysica Acta 990, 87–92.

Gowik U, Bräutigam A, Weber KL, Weber APM, Westhoff P. 2011. Evolution of C4 photosynthesis in the genus Flaveria—how many and which genes does it take to make C4? The Plant Cell 23, 2087–2105.

Gowik U, Westhoff P. 2011. The path from C3 to C4 photosynthesis. Plant Physiology 155, 56–63.

Griffiths H, Weller G, Toy LF, Dennis RJ. 2013. You’re so vein: bundle sheath physiology, phylogeny and evolution in C3 and C4 plants. Plant, Cell and Environment 36, 246–261.

Hari P, Mäkelä A, Korpilahti E, Holmberg M. 1986. Optimal control of photosynthesis, transpiration and photorespiration in different photosynthetic types in the genus Flaveria. Planta 186, 563–571.

Kapralov MV, Kubien DS, Andersson I, Filatov DA. 2011. Changes in Rubisco kinetics during the evolution of C4 photosynthesis in Flaveria (Asteraceae) are associated with positive selection on genes encoding the enzyme. Molecular Biology and Evolution 28, 1491–1503.

Katul G, Manzon S, Palmoth S, Oren R. 2010. A stomatal optimization theory to describe the effects of atmospheric CO2 on leaf photosynthesis and transpiration. Annals of Botany 105, 431–442.

Kocacinac F, McKown AD, Sage TL, Sage RF. 2008. Photosynthetic pathway influences xylem structure and function in Flaveria (Asteraceae). Plant, Cell and Environment 31, 1363–1376.

Krarl JP, Edwards GE, Ku MSB. 1991. Quantum yield of photosystem II and efficiency of CO2 fixation in Flaveria (Asteraceae) species under varying light and CO2. Australian Journal of Plant Physiology 18, 369–383.

Ku MSB, Wu J, Dai Z, Scott RA, Chu C, Edwards GE. 1991. Photosynthetic and photorespiratory characteristics of Flaveria species. Plant Physiology 96, 518–528.

Kubien DS, Whitney SM, Moore PV, Jesson LK. 2008. The biochemistry of Rubisco in Flaveria. Journal of Experimental Botany 59, 1767–1777.

Laisk A, Edwards GE. 2000. A mathematical model of C4 photosynthesis: the mechanism of concentrating CO2 in NADP-malic enzyme type species. Photosynthesis Research 66, 199–224.

Lloyd J, Farquhar GD. 1994. C-13 discrimination during CO2 assimilation by the terrestrial biosphere. Oecologia 99, 201–215.

Lüthi D, Le Floch M, Bereiter B, et al. 2008. High-resolution carbon dioxide concentration record 650,000–800,000 years before present. Nature 453, 379–382.

Manzoni S, Vico G, Katul G, Fay PA, Polley W, Palmroth S, Porporato A. 2011. Optimizing stomatal conductance for maximum carbon gain under water stress: a meta-analysis across plant functional types and climates. Functional Ecology 25, 456–467.

Manzoni S, Vico G, Palmroth S, Porporato A, Katul G. 2013. Optimization of stomatal conductance for maximum carbon gain under dynamic soil moisture. Advances in Water Resources 62, 90–105.

McKown AD, Dengler NG. 2007. Key innovations in the evolution of Kranz anatomy and C4 vein pattern in Flaveria (Asteraceae). American Journal of Botany 94, 382–399.

McKown AD, Moncalvo JM, Dengler NG. 2005. Phylogeny of Flaveria (Asteraceae) and inference of C4 photosynthesis evolution. American Journal of Botany 92, 1911–1928.

Monson RK. 1989. The relative contribution of reduced photorespiration, and improved water- and nitrogen-use efficiencies, to the advantages of C3–C4 intermediate photosynthesis in Flaveria. Oecologia 80, 215–221.

Monson RK, Jaeger CH. 1991. Photosynthetic characteristics of the C3–C4 intermediate Flaveria floridiana J.R. Johnson measured in its natural habitat. Evidence of the advantages to C3–C4 photosynthesis at high leaf temperatures. American Journal of Botany 78, 795–800.

Morison JIL, Gifford RM. 1983. Stomatal sensitivity to carbon dioxide and humidity: a comparison of two C3 and two C4 grasses. Plant Physiology 71, 789–796.

Osborne CP, Sack L. 2012. Evolution of C4 plants: a new hypothesis for an interaction of CO2 and water relations mediated by plant hydraulics. Philosophical Transactions of the Royal Society B: Biological Sciences 367, 583–600.

Rawson HM, Begg JE, Woodward RG. 1977. The effect of atmospheric humidity on photosynthesis, transpiration and water use efficiency of leaves of several plant species. Planta 134, 5–10.

Ripley BS, Cunniff J, Osborne CP. 2013. Photosynthetic acclimation and resource use by the C3 and C4 subspecies of Allotrope semilamata in low CO2 atmospheres. Global Change Biology 19, 900–910.

Sage RF. 2004. The evolution of C4 photosynthesis. New Phytologist 161, 341–370.

Sage RF, Sage TL, Kocacinac F. 2012. Photorespiration and the evolution of C4 photosynthesis. Annual Review of Plant Biology 63, 19–47.

Sudderth EA, Muhaidat RM, McKown AD, Kocacinac F, Sage RF. 2007. Leaf anatomy, gas exchange and photosynthetic enzyme activity in Flaveria kochiana. Functional Plant Biology 34, 118–129.

Taiz L, Zeiger E. 2010. Plant physiology , 5th edn. Sunderland, MA: Sinauer Associates.

Taylor SH, Ripley BS, Woodward FI, Osborne CP. 2011. Drought limitation of photosynthesis differs between C3 and C4 grass species in a comparative experiment. Plant, Cell and Environment 34, 65–75.

Taylor SH, Franks P, Hulme S, Spiggs E, Christin P-A, Edwards E, Woodward I, Osborne C. 2012. Photosynthetic pathway and ecological adaptation explain stomatal trait diversity amongst grasses. New Phytologist 193, 387–396.

Vico G, Manzon S, Palmoth S, Wei M, Katul G. 2013. A perspective on optimal leaf stomatal conductance under CO2 and light co-limitations. Agricultural and Forest Meteorology 182–183, 191–199.

Vico G, Porporato A. 2008. Modelling C3 and C4 photosynthesis under water-stressed conditions. Plant and Soil 313, 187–203.

Vogan PJ, Frohlich MW, Sage RF. 2007. The functional significance of C3–C4 intermediate traits in Heliotropium L. (Boraginaceae): gas exchange perspectives. Plant, Cell and Environment 30, 1337–1345.

Vogan PJ, Sage RF. 2011. Water-use efficiency and nitrogen-use efficiency of C3–C4 intermediate species of Flaveria Juss. (Asteraceae). Plant, Cell and Environment 34, 1415–1430.

von Caemmerer S. 1989. A model of photosynthetic CO2 assimilation and carbon-isotope discrimination in leaves of certain C3–C4 intermediates. Planta 176, 465–474.

von Caemmerer S. 2000. Biochemical models of leaf photosynthesis. Collingwood, Victoria: CSIRO.

von Caemmerer S. 2013. steady-state models of photosynthesis. Plant, Cell and Environment 36, 1617–1830.

Way DA. 2012. What lies between: the evolution of stomatal traits on the road to C4 photosynthesis. New Phytologist 193, 291–293.