C₂ photosynthesis generates about 3-fold elevated leaf CO₂ levels in the C₃–C₄ intermediate species *Flaveria pubescens*

Olav Keerberg¹,*, Tiit Pärnik¹, Hiie Ivanova¹, Burgund Bassünner² and Hermann Bauwe³,*

¹ Department of Plant Physiology, Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, 51014 Tartu, Estonia
² Center for Conservation and Sustainable Development, Missouri Botanical Garden, St. Louis, MO 63166-0299, USA
³ Department of Plant Physiology, University of Rostock, 18051 Rostock, Germany

* To whom correspondence should be addressed. E-mail: hermann.bauwe@uni-rostock.de; olav.keerberg@emu.ee

Received 9 January 2014; Revised 24 April 2014; Accepted 1 May 2014

Abstract

Formation of a photorespiration-based CO₂-concentrating mechanism in C₂–C₄ intermediate plants is seen as a prerequisite for the evolution of C₄ photosynthesis, but it is not known how efficient this mechanism is. Here, using *in vivo* Rubisco carboxylation-to-oxygenation ratios as a proxy to assess relative intraplastidial CO₂ levels is suggested. Such ratios were determined for the C₃–C₄ intermediate species *Flaveria pubescens* compared with the closely related C₃ plant *F. cronquistii* and the C₄ plant *F. trinervia*. To this end, a model was developed to describe the major carbon fluxes and metabolite pools involved in photosynthetic–photorespiratory carbon metabolism and used quantitatively to evaluate the labelling kinetics during short-term ¹⁴CO₂ incorporation. Our data suggest that the photorespiratory CO₂ pump elevates the intraplastidial CO₂ concentration about 3-fold in leaves of the C₃–C₄ intermediate species *F. pubescens* relative to the C₃ species *F. cronquistii*.

Key words: ¹⁴CO₂ labelling, C₃–C₄ intermediate plants, carbon-concentrating mechanism; *Flaveria*, glycine decarboxylation, photorespiration, photosynthesis.

Introduction

Land plants form three major classes characterized by specific modes of photosynthetic CO₂ assimilation. In C₃ plants, CO₂ enters metabolism directly via ribulose 1,5-bisphosphate (RubP) carboxylase/oxygenase (Rubisco). In the mesophyll of C₄ plant leaves and in CAM (crassulacean acid metabolism) plants, CO₂ is initially fixed by phosphoenolpyruvate carboxylase. The resulting four-carbon (C₄) compounds are decarboxylated in the Rubisco-containing bundle-sheath of C₄ plants (Hatch and Slack, 1970) or become stored in the vacuoles of CAM plants for daytime decarboxylation and refixation of the released CO₂ by Rubisco (Lüttge, 2004). Both modifications to the C₃ mode of CO₂ assimilation are adaptations to specific environmental conditions such as low CO₂ or water availability. While C₄ plants represent only about 3% of all land plant species, they dominate nearly all grasslands in the tropics, subtropics, and warm temperate zones (Sage, 2004). They also include highly productive crops, such as corn and sugar cane, and there is much interest to introduce yield-relevant features of C₄ photosynthesis into C₃ crops.

Given the ecological and agricultural significance of C₄ plants, it is important to understand how they evolved and what were the crucial steps in this process. A number of studies have shown that the evolution of C₄ photosynthesis was not a unique event but occurred at least 66 times during the past 35 million years (Sage, 2004; Sage *et al.*, 2012). Among these plant lineages, the small genus *Flaveria* (Yellowtops) has received particular attention because it includes species with CO₂ assimilation modes ranging from C₃ via a broad range...
of C₃–C₄ intermediate species to C₄ (Powell, 1978; Apel and Maass, 1981; Ku et al., 1983; Bauwe, 1984). Notably, extant *Flaveria* C₃–C₄ intermediate species represent true evolutionary intermediates between C₃ and C₄ photosynthesis (Kopriba et al., 1996; McKown et al., 2005). Major physiological features of such plants are low apparent photorespiration (Apel and Maass, 1981; Holaday et al., 1982, 1984) in combination with an enhanced refixation of photorespiratory CO₂ (Holbrook et al., 1985; Bauwe et al., 1987) and high glycine accumulation (Holaday and Chollet, 1983, 1984).

Mechanistically, corresponding to the distribution of the photorespiratory enzyme glycine decarboxylase (GDC) in leaves of C₃ plants (Ohnishi and Kanai, 1983), these specific characteristics are closely related to a confinement of GDC activity to the leaf bundle sheath (Hylton et al., 1988; Moore et al., 1988). Based on these and other data, it was hypothesized that C₃–C₄ intermediate species reduce apparent photorespiration by an efficient re-fixation of photorespired CO₂ in the bundle sheath (Monson et al., 1984; Edwards and Ku, 1987; Rawsthorne, 1992). This initial focus on the importance of CO₂ re-fixation was later extended by the hypothesis that the confinement of glycine decarboxylase could result in a concentration of CO₂ in the bundle sheath of C₃–C₄ intermediate plants (von Caemmerer, 1989; Monson and Rawsthorne, 2000). Today, such a mechanism, in which photorespiratory glycine serves as a vehicle to move ‘CO₂’ from the mesophyll to the GDC-containing bundle sheath, is seen as a crucial step during the evolution of C₄ photosynthesis (Bauwe, 2011; Sage et al., 2012). In other words, the multiple evolution of C₄ photosynthesis might have been triggered by and possibly even required the preceding presence of a much simpler CO₂ concentration system than the C₄ cycle, based on relatively small alterations to the high-flux photorespiratory glycine metabolism.

This hypothesis is now widely accepted and the genetic alterations necessary to restrict photorespiratory GDC activity to the bundle sheath are being unravelled (Wiludda et al., 2012; Schulze et al., 2013). On the other hand, it is not known how efficient this photorespiratory CO₂ pump could be. Here, ¹⁴CO₂ incorporation studies designed to obtain an estimate of the *in vivo* rates of the two Rubisco-catalysed reactions in the C₃–C₄ species *Flaveria pubescens* relative to the control C₃ species *Flaveria cronquistii* are reported. The ratio of these reactions, carboxylation versus oxygenation of RuBP, is co-determined by kinetic parameters of Rubisco and by the CO₂/O₂ concentration ratio (Laing et al., 1974; Peisker, 1974; Farquhar et al., 1980). Hence, a higher *in vivo* carboxylation/oxygenation ratio in *F. pubescens* relative to a control C₃ species would not only indicate an elevated CO₂/O₂ concentration ratio but also allow quantifying the efficiency of the photorespiratory CO₂ pump.

**Materials and methods**

*Plant growth and ¹⁴C labelling*

*Flaveria cronquistii* A.M. Powell (C₃), *Flaveria pubescens* Rydberg (C₃–C₄), and *Flaveria trinervia* (Spreng.) C. Mohr (C₄) were grown in soil in a controlled environment chamber at 28/22 °C (day/night) and 250–300 µmol photons m⁻² s⁻¹ at a photon period of 16h. Fully expanded leaves excised from 40–60-d-old plants were fixed by thin wires in a frame positioned in a purpose-built fast-acting ¹⁴CO₂ labelling device (Pärnik et al., 1987). Leaves were pre-illuminated at 30 Pa ¹³CO₂ and 210 kPa O₂ for 10–15 min at about 1200 µmol photons m⁻² s⁻¹ and 25 °C to ensure maximum stomata opening and achievement of the steady-state rate of photosynthesis. Plants were then exposed to ¹⁴CO₂ (2000 MBq mmol⁻¹) for 0.6, 1.2, 2.4, 5, 15, 60, 120, and 360 s at the same concentrations of CO₂ and O₂, temperature and light as applied during pre-illumination. At the given time points, within 0.1 s, the leaf samples were automatically transferred into boiling 80% ethanol. ¹⁴CO₂ incorporation was linear over the whole experiment. All experiments were performed in triplicate (three individual plants in three consecutive days, resulting in three leaf samples per time-point for each species).

*Metabolite analysis*

All leaf samples were individually extracted as described before (Värk et al., 1968) with slight modifications. After 2 min in boiling 80% ethanol, the samples were extracted for 15 min at 86 °C with 5 ml of 80% ethanol (twice) and 20% ethanol (once). All four ethanolic fractions were combined. The remaining samples were then further extracted for 15 min at 86 °C with 5 ml 96% ethanol acidified with 3 drops of 3 N HCl. The two extracts were separately (to avoid the hydrolysis of disaccharides) dried at 37 °C, individually re-dissolved in 5 ml H₂O each and cleared by centrifugation. The supernatants were combined, dried as above, and the metabolites re-dissolved in 1 ml H₂O. This final extract was used to determine total extractable radioactivity, radioactivity in amino acids (AAA 339 analyzer, Mikrotechna, Czech Republic), and other metabolites by using two-dimensional paper chromatography. Residual radioactivity in the fully extracted, dried, and triturated leaf samples was determined by using a non-aqueous scintillation cocktail. These analytical methods including the protocol used for starch analysis were described in more detail elsewhere (Keerberg et al., 2011).

*Photosynthetic–photorespiratory gas exchange*

Rates of net and true photosynthesis, photorespiratory CO₂ evolution from the leaf, intracellular decarboxylation of early photosynthates, and rates of reassimilation of photorespiratory CO₂ were determined during steady-state photosynthesis by using standard gas-exchange measurement techniques in combination with a radiogasometric method described before (Pärnik and Keerberg, 1995, 2007). In short, this method is based on the analysis of time curves of ¹⁴CO₂ evolution from labelled photosynthates in leaves previously exposed to ¹³CO₂. Photorespiration (210 kPa O₂) and day respiration (15 kPa O₂) were distinguished by measurement under different O₂ concentrations. Re-fixation ratios (D) of photorespiratory CO₂ were calculated from ¹⁴CO₂ evolution at the very high concentration of 3 kPa ¹³CO₂, where re-fixation of ¹⁴CO₂ evolved inside the cell is close to zero, relative to ¹⁴CO₂ evolution at air levels of ¹³CO₂.

*Modelling and data analysis*

From the radioactivity values for individual metabolites in combination with the specific radioactivity of the ¹⁴CO₂ fed to leaves, the amounts of carbon incorporated at the selected time points were calculated and plotted against the duration of feeding with ¹⁴CO₂. The amounts of carbon fixed in individual compounds were expressed in absolute (µmol C m⁻²) and relative (per cent of total carbon fixed) units. These experimental labelling curves contain the information about rates of all relevant carbon fluxes and corresponding metabolite pool sizes.

To extract this information on *Flaveria* photosynthetic–photorespiratory metabolism, the model shown in Fig. 1 was used. The model allows CO₂ incorporation into the reductive pentose
phosphate cycle (RPPC) either directly with rate $R_1$ or via the C$_4$ cycle with rate $R_0$. Total carbon flux through the photosynthetic cycle is denoted $R_2$. $R_3$ is the export rate of phosphorylated sugars into other pathways, for example, sucrose biosynthesis. $R_4$ denotes the rate of carbon efflux from the RPPC to the C$_3$ skeleton of C$_4$ acids, while $R_5$ describes the rate of accumulation of C$_4$-acids. In order to simplify calculations, metabolites were grouped into four pools: (i) pool ‘SP’ with sugar phosphates plus 3-phosphoglycerate, (ii) pool ‘Gly’ with metabolites of the two-carbon branch of the photosynthetic cycle, (iii) pool ‘Ser’ with metabolites of the three-carbon branch of the photosynthetic cycle, and (iv) pool ‘C$_3$’ with malate and aspartate. Each of these four pools comprises two metabolic sub-pools with different labelling kinetics, for example, photosynthetic pools with rapid turnover in peroxisomes and mitochondria (Gly-I and Ser-I with pools C$_4$ and C$_5$, respectively) or less mobility in the cytosol and chloroplasts (Gly-II and Ser-II with pools C$_4$ and C$_5$, respectively). At steady-state photosynthesis, these pools are in diffusional equilibrium with exchange rates $R_1$ and $R_5$, respectively. At the glycine-serine conversion step, one molecule of CO$_2$ is released per serine molecule formed, corresponding to a glycine decarboxylation rate of $R_7/4$. The resulting CO$_2$ is re-fixed in the RPPC or the C$_4$ cycle or escapes from the leaf. The extent of re-fixation is described by the re-fixation coefficient $D$, which was experimentally determined as described above.

Formally, the metabolic model is described by the four analytical functions shown as equations 1–4, one for each major metabolic pool (similar to Keerberg et al., 2011). To determine individual pool sizes $C_i$ and carbon fluxes $R_i$, the experimental values of the radioactivity of sugar phosphates, metabolites of the glycine and serine branches of the photosynthetic pathway, and of C$_4$-acids were simultaneously fitted to these functions by multi-component non-linear regression analysis. These functions also consider the time-dependent dilution of the applied tracer CO$_2$ by unlabelled photosynthetic CO$_2$, which is important particularly at the start of tracer feeding under steady-state photosynthesis. A more detailed explanation of these functions is provided in the Supplementary data at JXB online.

**Results and discussion**

The analysis of in vivo Rubisco carboxylation and oxygenation rates is not trivial. Potentially, such data can be extracted from gas exchange experiments (Pärnik and Keerberg, 1995), but this approach is biased by limited knowledge of the internal diffusion pathways for CO$_2$ and O$_2$. Bias becomes even stronger at a varying intercellular distribution of photosynthetic tasks, such as the operation of CO$_2$-concentrating mechanisms. Assuming that there is no large variation in the plastidial O$_2$ concentrations (Tolbert et al., 1995), it should be possible approximately to assess the efficiency of the photosynthetic CO$_2$ pump in C$_3$–C$_4$ intermediate plants by the quantification of carbon fluxes through the individual routes of the photosynthetic-photosynthetic biochemical network. Speed and complexity of the biochemical processes involved require fast and, consequently, sensitive labelling techniques using $^{14}$CO$_2$ as a tracer in combination with model-based data analysis.

For our study, three *Flaveria* species were used, *F. cronquistii* (C$_3$), *F. pubescens* (C$_3$–C$_4$ intermediate), and *F. trinervia* (C$_4$). These species have previously been examined for their photosynthetic types (Apel and Maass, 1981; Ku et al., 1983; Rumpho et al., 1984), kinetic properties of Rubisco (Bauwe et al., 1984; Wessinger et al., 1989; Kubien et al., 2008), and phylogenetic position within the genus (Powell, 1978; Kopriwa et al., 1996; McKown et al., 2005). These studies include the observation (Bassüner et al., 1984; Monson et al., 1986) that C$_3$–C$_4$ intermediate *Flaveria* species fix a small fraction of CO$_2$ via the C$_4$ pathway ($R_6$ in the model shown in Fig. 1) while most of the CO$_2$ enters metabolism directly via the RPPC ($R_1$ in Fig. 1). It was not our intention to perform a comprehensive re-analysis of photosynthetic-photosynthetic carbon
metabolism of these species. Instead, we wanted to focus on the quantification of key fluxes including control data confirming adequate fidelity of our approach.

Building upon previous studies (Pärnik et al., 1987; Keerberg et al., 2011), the model schematically shown in Fig. 1 was developed which embraces, in a generalized form, all the relevant information that is necessary to determine Rubisco carboxylation/oxygenation ratios in vivo. It considers time- and flux-dependent changes in the tracer’s specific radioactivity at all nodes of the network and allows the separation of high- and low-turnover pools of key metabolites of photosynthetic CO2 and photorespiratory O2 fixation. In order to simplify the model and make it as robust as possible, the metabolically related metabolites of the four major pathways were combined into four pools, each of which is described by a labelling function \( P(t,C_i,R_i) \) shown as equations 1–4.

\[
P(SP) = S_S \left[ C_1 E_A(t,C_1,R_S) + C_6 E_1(t,C_1,C_6,R_S) \right]
\]

\[
P(Gly) = S_S \left[ C_2 E_1(t,V_1,C_2,R_2) + C_4 E_1(t,V_2,C_4,R_3) \right]
\]

\[
P(Ser) = S_S \left[ C_3 E_1(t,V_4,C_3,0.75(R_2 - R_3)) + C_5 E_1(t,V_5,C_5,0.75R_3) + V_E(t,V_5,0.75R_3) \right]
\]

\[
P(C_4) = 0.25S_C C_7 E_A(t,0.25C_7,R_C) + S_S \left[ 0.75C_7 E_1(t,V_7,0.75C_7,R_C) + E_E(t,V_5,0.75R_6) \right]
\]

Essentially, these four functions describe the time dependence of the radioactivity \( P \) incorporated under steady-state conditions into each of the four major model components sugar phosphates plus 3-phosphoglycerate [equation (1); SP-I plus SP-II], the glycine branch [equation (2); Gly-I plus Gly-II] and the serine branch [equation (3); Ser-I plus Ser-II] of the photorespiratory pathway, and the \( C_4 \) pathway [equation (4); \( C_4 \)]. \( S_S \) and \( S_C \) are time-dependent functions that describe changes in the specific radioactivity of CO2 fixed in the RPPC and the \( C_4 \) pathways, respectively. Functions \( P(SP), P(Gly), P(Ser), \) and \( P(C_4) \) were simultaneously fitted to experimental data points collected over a time scale from 0.6 to 360 s during steady-state photosynthesis. Quantitative values for carbon fluxes \( R_i \) between the sub-pools directly involved in photosynthetic CO2 fixation and photorespiration, for example, from SP-I (pool size \( C_1 \)) via Gly-I (pool size \( C_2 \)) to Ser-I (pool size \( C_3 \)), were calculated by multi-component non-linear regression analysis.

Figure 2 demonstrates that the model approximations for all four major metabolite pools represented by the model fit
very well to the experimental data points. This includes initial CO₂ fixation by the C₄ pathway in *F. trinervia* in combination with final refixation of CO₂ released from C₄ acids by the RPPC as well as the ‘glycine anomaly’ of the C₃–C₄ intermediate plant *F. pubescens*. As mentioned in the Introduction, the specific alterations to glycine metabolism of C₃–C₄ intermediate plants are due to a specific distribution of photorespiratory GDC activity (Rawsthorne, 1992), which represents the enzymatic backbone of the photorespiratory CO₂ pump.

Another apparent feature is the overlap of primary and secondary labelling kinetics, which is best seen with the C₄ acids but also within the glycine and serine branches of the photorespiratory pathway (Keerberg *et al.*, 2011). In the case of the C₄ acids, the complex labelling kinetics results from direct CO₂ fixation (*R₆* in Fig. 1), secondary labelling of carbons 1–3 by the synthesis of phosphoenolpyruvate from RPPC intermediates (via phosphoglycerate mutase and enolase; *R₇*), and export as a metabolically less mobile pool (probably to the vacuole; *R₈*). Also, two metabolic pools with different labelling kinetics exist in both branches of the photorespiratory pathway. This is because one fraction each (Gly-II and Ser-II with pools *C₄* and *C₅*, respectively) is present in cellular compartments that do not directly contribute to photorespiratory reactions. These fractions show a lower turnover than the photorespiratory most active pools (Gly-I and Ser-I with pools *C₂* and *C₃*, respectively). At steady-state photosynthesis, the pools equilibrate pairwise with exchange rates *R₃* and *R₄*. To consider such effects, and specifically calculate fluxes between metabolite pools directly involved in CO₂ fixation and photorespiration, the model allows overlapping pools with different labelling kinetics to be separated by component analysis. Figure 3 provides examples of how the sequestration of metabolites into different pools was quantified and how the separation of primary and secondary labelling was achieved in the case of *F. pubescens*. The

---

**Fig. 3.** Examples for the model-based separation of fast- and slow-turnover pools in the ‘Gly’ and ‘Ser’ branches of the photorespiratory pathway and for primary versus secondary labelling and accumulation of C₄ acids. All data are for *F. pubescens*. 

---
example data display carbon incorporation into high- (Gly-I and Ser-I) and low-turnover (Gly-II and Ser-II) pools within the glycine and serine branches of the photorespiratory pathway. They also demonstrate the quantitative separation of the ‘active’ C₄ carbon pool of C₄ acids from label appearing in carbon atoms 1–3 and in C₄ acids exported to the vacuole. Collectively, these data show that the chosen model is an adequate tool for the calculation of fluxes through the major routes of photosynthetic CO₂ fixation from quantitative ¹⁴CO₂ labelling data.

The relevant fluxes are summarized in Table 1 and complemented by results from radiogasometric measurements performed in parallel with the same set of plants. These independent data show rates of true photosynthesis, total decarboxylation, and photorespiratory CO₂ evolution. They allowed calculating the extent to which photorespiratory CO₂ is re-fixed.

CO₂ can become incorporated into the RPPC either directly with rate $R_1$ or indirectly via the C₄ pathway with rate $R_6$. The sums $R_1 + R_6$ then represent total CO₂ incorporation from external sources and show an increasing contribution by the C₄ cycle, very low in F. cronquistii, low in F. pubescens, and, as expected, very high in F. trinervia. These total influx rates correspond reasonably well to directly measured rates for true photosynthesis $P_T$, which provides a strong argument for the soundness of all other flux calculations. Higher values for $P_T$ (C₃<C₃–C₄<C₄) go together with increased rates of sucrose formation ($R_8$; directly measured in Table 1) and C₄ acid accumulation as end-products ($R_8$). Moreover, the photosynthetically active pools of C₄ acids ($C_T$; not listed in Table 1) increased from 13 ± 1 (C₃) via 57 ± 19 (C₃–C₄) to 161 ± 39 µmol C m⁻² (C₄). It is important to note that the increase of C₄ cycle activity from F. cronquistii to F. pubescens (5.8% to 8.3% of $P_T$, calculated as $R_6 - R_4$) is only very small in comparison with the activity of the C₄ cycle in F. trinervia (81.7% of $P_T$). This suggests that CO₂ accumulation occurs mainly by glycine-shuttling and less by C₄ cycle activity in the bundle sheath of F. pubescens.

Carbon flux through the glycolate cycle, $R_2$, is stoichiometrically related to the rate of RuBP oxygenation, $R_2/2$. As a result of the operation of CO₂-concentrating mechanisms in F. pubescens and in F. trinervia, photorespiration-related fluxes become distinctly lower from C₃ towards C₄ metabolism. To determine the true rates of RuBP carboxylation, in addition to the sum of $R_1$ and $R_6$, it was necessary to consider the refixation of CO₂ generated from internal sources. In C₃ and C₃–C₄ plants, photorespiration is the dominating internal source of CO₂ during photosynthesis $R_1$ is stoichiometrically related to photorespiratory glycine decarboxylation as $R_1/4$, because one molecule of CO₂ is released per one molecule of serine formed from two glycine molecules. The extent to which refixation occurs must be separately determined. This was done by radiogasometric measurements (Pärnik and Keerberg, 1995, 2007), which allowed direct quantification of the sum DEC of photorespiratory glycine decarboxylation plus C₄ acid decarboxylation plus minor CO₂ releasing processes. It is reasonable to assume that all fractions of internally generated CO₂ are re-assimilated with the same efficiency. In combination with the rate $R_0$ of CO₂ losses from the leaf (simplifying referred to as photorespiratory CO₂ evolution), this assumption allows assessing the partitioning $D$ between re-fixation and loss of CO₂ from the leaf. The calculated total rates with which Rubisco fixes CO₂ arriving by diffusion from the stomata ($R_1$), from decarboxylation in the C₄ cycle ($R_4$), and from photorespiration ($D^* R_2/4$) were related to RuBP oxygenation rates ($R_2/2$). The comparison

### Table 1. Carbon fluxes in photosynthetic–photorespiratory carbon metabolism of Flaveria species

Values marked with an asterisk represent means ±SE from three measurements on different plants by using a radiogasometric method (Pärnik and Keerberg, 2007). All other values were calculated as means ±SE by multi-component non-linear regression analysis from the time-course of ¹⁴C-incorporation (simultaneous fit to equations 1–4; labelling data from three independent experiments).

| Carbon fluxes | F. cronquistii | F. pubescens | F. trinervia |
|---------------|---------------|---------------|---------------|
| $P_T$ (%)     | µmol m⁻² s⁻¹  | µmol m⁻² s⁻¹  | µmol m⁻² s⁻¹  |
| $P_T$         | 3.76 ± 0.10   | 7.93 ± 0.70   | 10.37 ± 0.28  |
| $R_1$         | 3.82 ± 0.49   | 6.23 ± 0.07   | 7.96 ± 0.70   |
| $R_6$         | 0.32 ± 0.01   | 1.29 ± 0.32   | 16.3 ± 2.70   |
| $R_6$         | 0.43 ± 0.07   | 1.71 ± 0.03   | 21.6 ± 1.70   |
| $R_8$         | 0.10 ± 0.01   | 0.66 ± 0.06   | 8.3 ± 0.45    |
| $R_1 + R_6$   | 4.14 ± 0.49   | 7.52 ± 0.33   | 94.8 ± 9.5    |
| $R_1$         | 0.96 ± 0.02   | 2.11 ± 0.09   | 26.6 ± 5.6    |
| $R_6$         | 0.86 ± 0.02   | 1.58 ± 0.13   | 19.9 ± 1.8    |
| $R_4$         | 6.64 ± 0.25   | 3.66 ± 0.20   | 46.2 ± 2.5    |
| $R_2/2$       | 1.66 ± 0.06   | 0.92 ± 0.05   | 11.6 ± 0.6    |
| $R_2/2$       | 2.18 ± 0.08   | 1.84 ± 0.05   | 23.2 ± 2.1    |
| $D^*$         | 1.35 ± 0.05   | 0.16 ± 0.02   | 2.0 ± 0.03    |
| $R_1/2$       | 3.3           | 1.8           | 1.3           |
| $R_1 + R_6 + D^* R_2/4$ | 4.8 | 8.3 | 10.5 |
| Mean relative CO₂ at Rubisco sites | 1.0 | 3.2 | 5.7 |
shows that the resulting in vivo carboxylation-to-oxygenation ratio of Rubisco is more than three times higher in F. pubescens relative to F. cronquistii under the same experimental conditions.

Rubisco from C₄ Flaveria species has a somewhat lower affinity to CO₂, but it is also known that Rubisco from C₃ and C₃-C₄ Flaveria species show more or less identical kinetics (Bauwe, 1984; Wessinger et al., 1989; Kubien et al., 2008). Since the oxygen compensation point of C₃ plants is only slightly above air levels (Tolbert et al., 1995), plastidal oxygen concentrations are probably close to air oxygen concentrations in F. cronquistii and F. pubescens but presumably also in the C₃ species F. trinervia. Therefore, in a comparison of these species, measurement of in vivo carboxylation-to-oxygenation ratios allows the calculation of the relative CO₂ concentration in chloroplasts. Considering the reported $K_m$ values of Rubisco for CO₂, which are even somewhat higher than steady-state internal CO₂ levels, our data suggest that the photorespiratory CO₂ pump elevates the mean intraplastidial CO₂ concentration during steady-state photosynthesis about 3-fold in leaves of the C₃-C₄ intermediate species F. pubescens relative to the C₃ species F. cronquistii. This is considered to be a sound estimate because small contributions from C₃ photosynthesis are balanced by the operation of a significant fraction of Rubisco at non-elevated CO₂ levels in the mesophyll of F. pubescens.

**Supplementary data**

Supplementary data can be found at JXB online.

Supplementary data. An explanation of the labelling functions of the model shown in Fig. 1 used for the quantitative analysis of the labelling kinetics.

**Acknowledgements**

We wish to acknowledge help during the experiments by Hille Keerberg. This work was supported by the Akademie der Wissenschaften der DDR, the Estonian Science Foundation (grants 4173 and 5989), the Estonian Ministry of Education and Research (IUT-8-3), the EU’s 7th Framework Programme (KBBE-2011-289582), the European Regional Fund (Center of Excellence in Estonian Science Foundation (grants 4173 and 5989), the Estonian Ministry of Environmental Adaptation), and by the Deutsche Forschungsgemeinschaft (KBBE-2011-289582), the European Regional Fund (Center of Excellence in Environmental Adaptation), and by the Deutsche Forschungsgemeinschaft (FOR 1186).

**References**

Apel P, Maass I. 1981. Photosynthesis in species of Flaveria. CO₂ concentration compensation, O₂ influence on photosynthetic gas exchange and δ¹³C values in species of Flaveria (Asteraceae). Biochimie et Physiologie der Pflanzen 176, 396–399.

Bassünner B, Keerberg O, Bauwe H, Pärnik T, Keerberg H. 1984. Photosynthetic CO₂ metabolism in C₃-C₄ intermediate and C₃ species of Flaveria (Asteraceae). Biochimie et Physiologie der Pflanzen 179, 631–634.

Bauwe H. 1984. Photosynthetic enzyme activities and immunofluorescence studies on the localization of ribulose-1,5-bisphosphate carboxylase/oxygenase in leaves of C₃, C₄, and C₃-C₄ intermediate species of Flaveria (Asteraceae). Biochimie et Physiologie der Pflanzen 179, 253–268.

Bauwe H. 2011. Photorespiration: the bridge to C₄ photosynthesis. In: Raghavendra AS, Sage R, eds. C₄ photosynthesis and related CO₂ concentrating mechanisms, Vol. 32. New York: Springer Science+Business Media BV, 81–108.

Bauwe H, Keerberg O, Bassünner R, Pärnik T, Bassünner H. 1987. Reassimilation of carbon dioxide by Flaveria (Asteraceae) species representing different types of photosynthesis. Planta 172, 214–218.

Edwards GE, Ku MSB. 1987. Biochemistry of C₃-C₄ intermediates. In: Hatch MD, Boardman NK, eds. The biochemistry of plants, Vol. 10. London: Academic Press, 275–325.

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

Hatch MD, Slack CR. 1970. Photosynthetic CO₂ fixation pathways. Annual Review of Plant Physiology 21, 141–162.

Holaday AS, Chollet R. 1983. Photosynthetic/photorespiratory carbon metabolism in the C₃-C₄ intermediate species, Moricandia arvensis and Panicum milioides. Plant Physiology 73, 740–745.

Holaday AS, Chollet R. 1984. Photosynthetic/photorespiratory characteristics of C₃-C₄ intermediate species. Photosynthesis Research 5, 307–323.

Holaday AS, Harrison AT, Chollet R. 1982. Photosynthetic/ photorespiratory CO₂ exchange characteristics of the C₃-C₄ intermediate species, Moricandia arvensis. Plant Science Letters 27, 181–189.

Holaday AS, Lee KW, Chollet R. 1984. C₃-C₄ intermediate species in the genus Flaveria: leaf anatomy, ultrastructure, and the effect of O₂ on the CO₂ compensation concentration. Planta 160, 25–32.

Holbrook GP, Jordan DB, Chollet R. 1985. Reduced apparent photorespiration by the C₃-C₄ intermediate species, Moricandia arvensis and Panicum milioides. Plant Physiology 77, 578–583.

Hylton CM, Rawsthorne S, Smith AM, Jones DA, Woolhouse HW. 1988. Glycine decarboxylase is confined to the bundle-sheath cells of leaves of C₃-C₄ intermediate species. Planta 175, 452–459.

Keerberg O, Ivanova H, Keerberg H, Pärnik T, Talts P, Gardeström P. 2011. Quantitative analysis of photosynthetic carbon metabolism in protoplasts and intact leaves of barley. Determination of carbon fluxes and pool sizes of metabolites in different cellular compartments. BioSystems 103, 291–301.

Kopriva S, Chu CC, Bauwe H. 1996. Molecular phylogeny of Flaveria as deduced from the analysis of nucleotide sequences encoding H-protein of the glycine cleavage system. Plant, Cell and Environment 19, 1028–1036.

Ku MSB, Monson RK, Littlejohn RO, Nakamoto H, Fischer DB, Edwards GE. 1993. Photosynthetic characteristics of C₃-C₄ intermediate Flaveria species. I. Leaf anatomy, photosynthetic responses to O₂ and CO₂ and activities of key enzymes in the C₃ and C₄ pathways. Plant Physiology 71, 944–948.

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

Laing WA, Ogren WL, Hageman RH. 1974. Regulation of soybean net photosynthetic CO₂ fixation by the interaction of CO₂, O₂, and ribulose 1,5-diphosphate carboxylase. Plant Physiology 54, 678–685.

Lütßge U. 2004. Ecophysiology of Crassulaceae Acid Metabolism (CAM). Annals of Botany 93, 629–652.

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, Edwards GE, Ku MSB. 1984. C₃-C₄ intermediate photosynthesis in plants. BioScience 34, 563–574.

Monson RK, Moore BD, Ku MSB, Edwards GE. 1986. Co-function of C₃- and C₄-photosynthetic pathways in C₃, C₄ and C₃-C₄ intermediate Flaveria species. Planta 166, 493–502.

Monson RK, Rawsthorne S. 2000. C₃-C₄ intermediate photosynthesis. In: Leegood RC, Sharkey TD, von Caemmerer S, eds. Photosynthesis, physiology and metabolism, Vol. 9. Dordrecht: Kluwer Academic Publishers, 553–550.

Moore BD, Monson RK, Ku MSB, Edwards GE. 1988. Activities of principal photosynthetic and photorespiratory enzymes in leaf mesophyll and bundle sheath protoplasts from the C₃-C₄ intermediate Flaveria ramosissima. Plant and Cell Physiology 29, 999–1006.
Ohnishi J, Kanai R. 1983. Differentiation of photorespiratory activity between mesophyll and bundle sheath cells of C4 plants. I. Glycine oxidation by mitochondria. Plant and Cell Physiology 24, 1411–1420.

Pärnik T, Keerberg O. 1995. Decarboxylation of primary and end-products of photosynthesis at different oxygen concentrations. Journal of Experimental Botany 46, 1439–1447.

Pärnik T, Keerberg O. 2007. Advanced radiogasometric method for the determination of the rates of photorespiratory and respiratory decarboxylations of primary and stored photosynthates under steady-state photosynthesis. Physiologia Plantarum 129, 34–44.

Pärnik T, Keerberg OF, Yurisma EY. 1987. Fast-acting exposure chamber for studying photosynthesis with C-14 CO2. Soviet Plant Physiology 34, 676–683.

Peisker M. 1974. A model describing the influence of oxygen on photosynthetic carboxylation. Photosynthetica 8, 47–50.

Powell AM. 1978. Systematics of Flaveria (Flaveriinae-Asteraceae). Annals of the Missouri Botanical Garden 65, 590–636.

Rawsthorne S. 1992. C3–C4 intermediate photosynthesis: linking physiology to gene expression. The Plant Journal 2, 267–274.

Rumpho ME, Ku MSB, Cheng SH, Edwards GE. 1984. Photosynthetic characteristics of C3–C4 intermediate Flaveria species. III. Reduction of photorespiration by a limited C4 pathway of photosynthesis. Plant Physiology 75, 993–996.

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

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

Schulze S, Mallmann J, Burscheidt J, Koczor M, Strebel M, Bawue H, Gowik U, Westhoff P. 2013. Evolution of C4 photosynthesis in the genus Flaveria: establishment of a photorespiratory CO2 pump. The Plant Cell 25, 2522–2535.

Tolbert NE, Benker C, Beck E. 1995. The oxygen and carbon dioxide compensation points of C3 plants - possible role in regulating atmospheric oxygen. Proceedings of the National Academy of Sciences, USA 92, 11230–11233.

Värk E, Keerber H, Keerberg O, Pärnik T. 1968. On the extraction of the products of photosynthesis by ethanol of different concentrations (in Russian). Proceedings of the Estonian Academy of Sciences (Biology) 17, 367–373.

von Caemmerer S. 1989. A model of photosynthetic CO2 assimilation and carbon-isotope discrimination in leaves of certain C3–C4 intermediates. Planta 178, 463–474.

Wessinger ME, Edwards GE, Ku MSB. 1989. Quantity and kinetic properties of ribulose 1,5-bisphosphate carboxylase in C3, C4, and C3–C4 intermediate species of Flaveria (Asteraceae), Plant and Cell Physiology 30, 665–671.

Wiludda C, Schulze S, Gowik U, Engelman S, Koczor M, Strebel M, Bawue H, Westhoff P. 2012. Regulation of the photorespiratory GLDPA gene in C4 Flaveria: an intricate interplay of transcriptional and posttranscriptional processes. The Plant Cell 24, 137–151.