High atmospheric CO₂ concentration causes increased respiration by the oxidative pentose phosphate pathway in chloroplasts

Introduction

Despite significant research efforts, the question of whether rising atmospheric CO₂ concentration (Cₐ) affects leaf respiration remains unanswered (González-Meler et al., 2004; Way et al., 2015; Dusenge et al., 2019). A large body of research conveys an entirely inconsistent picture including reports of both positive and negative responses. This may (inter alia) be due to methodological difficulties to disentangle overlapping CO₂ fluxes at the tissue level and an incomplete understanding of respiration at the metabolic level with a strong research focus on mitochondrial processes. Overall, leaf respiration has remained a major unknown from the metabolic to the Earth system level.

State-of-the-art isotope techniques enable analyses of specific metabolic fluxes (Ehlers et al., 2015; Wieloch et al., 2021b, 2022c; Xu et al., 2022). Recently, we reported two deuterium (D) fractionation signals (i.e. systematic variability in D abundance) in starch glucose of sunflower leaves (Wieloch et al., 2022a). A signal at glucose H₁ reflects hydrogen (H) isotope fractionation by chloroplast glucose-6-phosphate dehydrogenase (G6PD) and associated flux through the oxidative pentose phosphate pathway (OPPP; Fig. 1a). This anaplerotic pathway feeds pentose phosphates into the Calvin–Benson cycle (CBC), supplies NADPH, and releases CO₂. Another signal at glucose H₂ reflects H isotope fractionation by chloroplast phosphoglucone isomerase (PGI) and associated shifts of the reaction catalysed by PGI from kinetic to equilibrium conditions.

Here, these fractionations at glucose H₁ and H₂ are respectively expressed as:

\[
\delta D_1 = \frac{D_1}{D_{6S}} - 1 \quad \text{Eqn 1}
\]

and

\[
\delta D_2 = \frac{D_2}{D_{6R}} - 1 \quad \text{Eqn 2}
\]

where Dₙ denotes relative D abundances at specific carbon (C)-bound H atoms of glucose. In these equations, D abundances at glucose H⁶S and H⁶R are used as references because glucose H¹ and H⁶S and H² and H⁶R have the same precursors at the chloroplast triose-phosphate level, and H⁶S and H⁶R are not modified in the starch biosynthesis pathway (Wieloch et al., 2022a). In these notations, increases of δD₁ above zero reflect increases in anaplerotic flux into the CBC, whereas increases of δD₂ from negative to positive values reflect shifts of the PGI reaction from being on the side of fructose 6-phosphate (F6P), to being at equilibrium, to being on the side of glucose 6-phosphate (G6P) (Wieloch et al., 2022a).

Previously, we investigated these processes in leaves of sunflowers raised over 7–8 wk at Cₐ = 450 ppm (Wieloch et al., 2022a). We reported evidence against anaplerotic flux under these conditions. However, moving the plants into a low-Cₐ atmosphere for 2 d led to significant increases in δD₁ and δD₂, consistent with an increase in anaplerotic flux and a shift of the PGI reaction from kinetic to equilibrium conditions, respectively (see reanalysis of previous low-Cₐ results based on Eqns S1 and S2 in Supporting Information Notes S1). Related fractionation signals were also found in the starch derivative tree-ring glucose under drought (Wieloch et al., 2018, 2022b).

Here, I reanalyse our previously published data (Wieloch et al., 2022a) to assess how metabolism behaves after moving the plants into a high-Cₐ atmosphere for 2 d. I report that, as Cₐ increases from 450 to 1500 ppm, respiration by the OPPP in chloroplasts increases from 0 to ≈ 5% relative to the rate of net C assimilation. This is consistent with known regulatory properties of the pathway. Summarizing recent reports of metabolic fluxes in plant leaves, a picture emerges in which mitochondrial processes seem distinctly less important for overall respiration than the OPPPs in chloroplasts and the cytosol. My findings and regulatory properties of these pathways are consistent with observations of lower than expected increases of photosynthesis in response to increasing Cₐ. Reported advances in understanding leaf respiratory mechanisms may enable modelling and prediction of respiration effects (inter alia) on biosphere–atmosphere CO₂ exchange and plant performance under climate change.

Anaplerotic flux and associated respiration increase at high Cₐ

In contrast to Cₐ = 450 ppm, δD₁ is significantly greater than zero at Cₐ = 700 ppm (29%) and Cₐ = 1500 ppm (67%) (Fig. 1b; one-tailed one-sample t-test: P < 0.05, n = 5). This is consistent with significant anaplerotic flux into the CBC. By contrast, δD₂ exhibits low values of c. −427‰ at Cₐ ≥ 450 ppm (Fig. 1c), indicating that the PGI reaction remains stably removed from equilibrium on the side of F6P (cf. Wieloch et al., 2022a). The absence of a δD₂ response is remarkable, because anaplerotic flux was proposed to be controlled at the level of PGI (Sharkey & Weise, 2016). Accordingly, we previously observed simultaneous shifts of δD₁ and δD₂ for Cₐ shifts below 450 ppm (Fig. S1) (Wieloch et al., 2022a). Thus, the results...
suggest regulatory differences of the anaplerotic pathway for low and high \( C_a \) conditions.

In the light, chloroplast G6PD is inhibited by redox regulation via thioredoxin (Née et al., 2009), yet inhibition may be reversed allosterically by increasing concentrations of G6P (Cossar et al., 1984; Preiser et al., 2019). At medium \( C_a \), the PGI reaction in chloroplasts is strongly removed from equilibrium on the side of F6P, resulting in low [G6P]/[F6P] ratios and G6P concentrations (Dietz, 1985; Gerhardt et al., 1987; Kruckeberg et al., 1989; Schleucher et al., 1999). Low G6P concentrations are believed to restrict the anaplerotic flux (Sharkey & Weise, 2016). Towards low \( C_a \), G6P concentrations increase more than F6P concentrations, that is, the PGI reaction shifts towards equilibrium (Dietz, 1985). Towards high \( C_a \), [G6P]/[F6P] ratios remain low, yet F6P and G6P concentrations both increase along with net C assimilation (Dietz, 1985). Thus, towards low \( C_a \), G6P concentrations and anaplerotic flux increase due to regulation at PGI. By contrast, increases towards high \( C_a \) are not caused by regulation at PGI but probably by increases in net C assimilation and concomitantly increasing G6P concentrations.

**Estimation of anaplerotic flux and associated respiration at high \( C_a \)**

A previously published model describing H isotope fractionation by G6PD can be used to estimate anaplerotic flux into the CBC, associated respiration, and NADPH supply (Wieloch et al., 2022a). At \( C_a \approx 700 \) ppm, \( \approx 4.2\% \) of the G6P entering the starch biosynthesis pathway is diverted into the anaplerotic pathway, whereas it is \( \approx 9.4\% \) at 1500 ppm. Assuming 50% of all net assimilated C becomes starch (Sharkey et al., 1985), anaplerotic respiration proceeds at \( \approx 2\% \) and \( \approx 5\% \) relative to net C assimilation at \( C_a \approx 700 \) and 1500 ppm, respectively. These estimates are based on \( \delta D_1 \) signal strengths in starch glucose. At medium to high \( C_a \), the PGI reaction is on the side of F6P (Fig. 1c) (Dietz, 1985). To a degree, this prevents conversion of G6P (the site of signal introduction) back to F6P. F6P may leave the starch biosynthesis pathway via transketolase, causing signal washout (Wieloch et al., 2022a). At low \( C_a \), the PGI reaction is closer to or at equilibrium and signal washout can be expected to be significant (Notes S1) (Wieloch et al., 2022a). Thus, the G6PD fractionation model may significantly underestimate anaplerotic flux at low \( C_a \), whereas high-\( C_a \) estimates can be expected to be closer to actual values.

**Potential causes of lower than expected increases of photosynthesis in response to increasing \( C_a \)**

In \( C_3 \) plants, net C assimilation increases with increasing \( C_a \) (Drake et al., 1997; Ainsworth & Long, 2005). However, responses seen in free-air CO2 enrichment (FACE) experiments differ significantly among plant functional groups, with trees showing the strongest increase (Nowak et al., 2004; Ainsworth & Long, 2005).
Hence, some plant functional groups apparently come closer to theoretically possible increases calculated from Rubisco kinetics than others do (Long, 1991). As already shown herein, anaplerotic flux increases with increasing \( \frac{C_a}{C_i} \). Thus, respiration by the anaplerotic pathway can explain part of the lower than expected increase of net C assimilation by \( \frac{C_a}{C_i} \).

Another part may be explained by respiration by the cytosolic OPPP. Primary control of flux through this pathway is exerted at the level of its first enzyme, G6PD. In the light, cytosolic G6PD activity in potato leaf discs was shown to increase with increasing glucose concentration through \textit{de novo} enzyme synthesis (Hauschild & von Schaewen, 2003). In Arabidopsis rosettes, 88% of the glucose was shown to be in the vacuole and cytosol (Szecowka et al., 2013). As part of sucrose cycling, cytosolic hexokinase converts this glucose into G6P (Dancer et al., 1990; Xu et al., 2022). G6P-derived C can re-enter the CBC via the cytosolic OPPP (Eicks et al., 2002; Xu et al., 2022). This may explain why trees come closer to theoretically possible increases (Long, 1991). However, the additional C does not result in stronger increases in leaf soluble-sugar concentration. On the contrary, trees exhibit lower increases in leaf soluble-sugar concentration than other plant functional groups do (Ainsworth & Long, 2005). By contrast, trees exhibit the highest increase in dry matter accumulation (Ainsworth & Long, 2005). Thus, low increases in leaf soluble-sugar concentration are probably explained by high sink strengths of the relatively young and fast-growing trees studied in FACE experiments. In turn, low increases in leaf soluble-sugar concentration may cause low increases in respiration by both the plastidial and cytosolic OPPP (see earlier). This may explain why trees come closer to theoretically possible increases in net C assimilation in response to increasing \( \frac{C_a}{C_i} \) than other plant functional groups do. Overall, based on analyses and argumentation presented here, increases of net C assimilation by increasing \( \frac{C_a}{C_i} \) (including acclimation effects) may depend on plant sink strength and associated OPPP respiration.

OPPP flux in chloroplasts introduces a \( \delta D_1 \) signal in starch (see earlier). Similarly, OPPP flux in the cytosol of leaves can be expected to introduce a \( \delta D_1 \) signal in the glucosyl and fructosyl moieties of sucrose. These signals will be recorded in tree-ring cellulose because tree-ring cellulose is synthesized from starch and sucrose. However, signal interpretation will be complicated by several processes, including signal washout in chloroplasts (see earlier) and OPPP flux and triose phosphate cycling in the cytosol of tree-ring cells. Nevertheless, I believe these complications can be addressed, and I encourage the development of \( \delta D_1 \) signal analysis to retrieve information about leaf respiration and respiratory acclimation to increasing \( \frac{C_a}{C_i} \) from leaf, phloem, and tree-ring metabolites.

![Figure 2](image-url)  
**Fig. 2** Respiration at 5°C (\( R_5 \)) as a function of glucose concentration in illuminated needles of 33-yr-old Pinus banksiana. Dotted line, positive linear relationship between both variables (\( R^2 = 0.88, P < 10^{-6}, n = 15 \)), Red cross, outlier removed before regression analysis. Data collected from eight provenances (boreal to temperate origin, 44–55°N) grown in a common garden in Cloquet, MN, USA. Sun-exposed canopy branch sampled from four randomly selected trees per provenance at two dates, one in mid-November 1997 and one in mid-May 1998. \( R_5 \) measured in a laboratory at an atmospheric CO2 concentration of c. 380 ppm within 6 h after sampling by infrared gas analysers and cuvettes (LCA-3 and PLC-C; Analytical Development Co., Hoddesdon, UK). Note, respiration was shown to remain stable over several hours after sampling (Mitchell et al., 1999; Ow et al., 2008; Tjoelker et al., 2009). Glucose concentration measured using a high-performance liquid chromatograph (Waters Associates, Milford, MA, USA) equipped with a Sugar Pack I column and a refractive index detector (Waters 410) following published procedures (Pukacka & Pukacki, 1997). Soluble sugar extracted from dried needles used as starting material. Figure shows data published by Tjoelker et al. (2009). For further information on materials and methods, see Tjoelker et al. (2009).
A paradigm shift in the field of leaf day respiration?

In the past, research of leaf day respiration had a strong focus on mitochondrial processes. However, according to recent analyses of metabolic fluxes in leaves of Arabidopsis thaliana and Camelina sativa, mitochondrial respiration is relatively low (1–1.6% relative to the rate of net C assimilation) (Ma et al., 2014; Xu et al., 2022). By contrast, respiration by the cytosolic OPPP is ≈ 5% relative to the rate of net C assimilation in C. sativa leaves (Xu et al., 2022). Similarly, in sunflower leaves, respiration by the OPPP in chloroplasts is relatively high under both high (see earlier herein) and low C3 (Notes S1; Wieloch et al., 2021a, 2022a). These findings indicate that the OPPP may be more important for overall leaf day respiration than mitochondrial processes.

Competing interests
None declared.

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Data availability
The data supporting the findings of this study have been published previously (Tjoelker et al., 2009; Wieloch et al., 2022a).

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Deuterium abundance at glucose H1 and H2 of sunflower leaf starch.

Notes S1 Recalculation of previously reported estimates of flux through the plastidial anaplerotic pathway at low Ca.

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