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**Respiratory metabolism of illuminated leaves depends on CO<sub>2</sub>- and O<sub>2</sub>-conditions**

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**Abbreviations:** DHAP, dihydroxyacetone phosphate; Fd-GOGAT, ferredoxin-dependent glutamine-2-oxoglutarate aminotransferase; Fum, fumarate; Mal, malate; NMR, nuclear magnetic resonance; PDH, pyruvate dehydrogenase complex; PEPC, phosphoenolpyruvate carboxylase; Pyr, pyruvate; Succ, succinate; TCA, tricarboxylic acid.

1 **Abstract**

2

3 Day respiration is the process by which non-photorespiratory CO<sub>2</sub> is produced by illuminated  
4 leaves. The biological function of day respiratory metabolism is a major conundrum of plant  
5 photosynthesis research: since the rate of CO<sub>2</sub>-evolution is partly inhibited in the light, it is  
6 viewed as either detrimental to plant carbon balance or necessary for photosynthesis operation  
7 (e.g. in providing cytoplasmic ATP for sucrose synthesis). Systematic variations in day  
8 respiration rate under contrasting environmental conditions have been used to elucidate the  
9 metabolic rationale of respiration in the light. Using isotopic techniques, we show that both  
10 glycolysis and the TCA cycle activities are inversely related to the ambient CO<sub>2</sub>/O<sub>2</sub> ratio: day  
11 respiratory metabolism is enhanced under high photorespiratory (low-CO<sub>2</sub>) conditions. Such a  
12 relationship also correlates with the DHAP/Glc-6-P ratio, suggesting that photosynthetic  
13 products exert a control on day respiration. Thus, day respiration is normally inhibited by  
14 phosphoryl (ATP/ADP) and reductive (NADH/NAD) poise, but up-regulated by  
15 photorespiration. Such an effect may be related to the need for NH<sub>2</sub>-transfers during the  
16 recovery of photorespiratory cycle intermediates.

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## 1 **Introduction**

2  
3 It is now seventy years since Krebs and Johnson proposed the mechanism by which pyruvic  
4 acid is oxidised to CO<sub>2</sub>, and now called the 'Krebs cycle' or TCA cycle (1,2). While the  
5 basics of the metabolic reactions involved in leaf respiration are known, intense efforts are  
6 still currently devoted to elucidating the regulation of the TCA cycle (and, more generally, of  
7 day respiration) in illuminated leaves (for a recent review, see ref. 3).

8 Leaf day respiration (non-photorespiratory CO<sub>2</sub> evolution in the light) is an essential  
9 metabolic pathway that accompanies photosynthetic CO<sub>2</sub> assimilation and photorespiration. It  
10 is widely accepted that leaf respiration is partly inhibited in the light when compared to  
11 darkness (4). This acceptance is based on several strong lines of evidence, ranging from gas-  
12 exchange to molecular studies (for a review, see ref. 4): (i) the inhibition is thought to cause  
13 the light-enhanced dark respiration (5); (ii) the pyruvate dehydrogenase (PDH) is down-  
14 regulated in the light (6,7); (iii) the metabolic flux through the TCA cycle in the light is  
15 reduced in both extracted mitochondria (8) and intact leaves (9,10); (iv) mitochondria  
16 experience high ATP/ADP and NADH/NAD<sup>+</sup> ratios in the light that inhibit NAD-dependent  
17 isocitrate dehydrogenase (11); and (v) carbohydrate molecules such as sucrose and glucose  
18 are prevented from entering glycolysis (9), due to a modification of phosphofructokinase  
19 activity by the allosteric effector Fru-2,6-bisphosphate (12). Nevertheless, not all leaf cells are  
20 photosynthetic (e.g. most epidermal cells, phloem and xylem) so that some 'heterotrophic'  
21 background respiration in the light is expected, but its contribution is minor.

22 Although inhibited by light, day respiration is critical for plant growth and leaf N  
23 assimilation, as it provides ATP for Suc synthesis and TCA cycle intermediates (e.g. 2-  
24 oxoglutarate and oxaloacetate) for ammonium assimilation and amino-acid synthesis (13).  
25 Thus for many years, the down-regulation of day respiration and of the TCA-cycle in the light  
26 has been viewed as a perplexing phenomenon. It may be argued that the partial inhibition of

1 day respiration comes from a balance between two metabolic constraints: the energy  
2 requirement for Suc synthesis and the minimal competition between glycolysis and Suc  
3 synthesis for improved carbon gain. However, day respiration is additionally affected by other  
4 metabolic processes such as O<sub>2</sub> assimilation (photorespiration) (14), the rate of which depends  
5 on the internal CO<sub>2</sub>/O<sub>2</sub> ratio. The photorespiratory cycle leads to Gly oxidative  
6 decarboxylation in the mitochondrion that supposedly gives rise to a large NADH/NAD<sup>+</sup>  
7 ratio, that in turn inhibits certain respiratory mitochondrial enzymes *in vivo* (11).  
8 Photorespiration is thus assumed to down-regulate day respiratory CO<sub>2</sub>-evolution.  
9 Nevertheless, increased photorespiration rates could require more Glu cycling to provide  
10 amino groups for Gly synthesis in the peroxisomes. This higher demand might in turn require  
11 an increase in 2-oxoglutarate and Glu synthesis, and thus a higher day respiratory rate. The  
12 rationale of the metabolic homeostasis between day respiration and photorespiration are  
13 therefore currently uncertain.

14 To clarify the regulation of day respiration in illuminated leaves and its interactions  
15 with photorespiration, we have investigated the effect of the carboxylation-to-oxygenation  
16 ratio on day respiratory metabolic fluxes using isotopic <sup>12</sup>C/<sup>13</sup>C spectrometry and <sup>13</sup>C and <sup>31</sup>P  
17 nuclear magnetic resonance (NMR). The results presented in this paper show that, while  
18 respiratory CO<sub>2</sub> evolution is always inhibited in the light when compared to the dark, the  
19 metabolic flux associated with the TCA cycle is inversely related to net CO<sub>2</sub> assimilation and  
20 this correlates with changes in phosphorylated metabolites levels. In addition, <sup>13</sup>C-distribution  
21 after labeling shows a larger commitment towards TCA intermediates and Glu as  
22 photorespiration increases. These findings, which are consistent with a role of day respiration  
23 in sustaining photorespiratory N cycling and perhaps nitrate assimilation, have important  
24 implications, ranging from the improvement of nitrogen use efficiency to the understanding of  
25 leaf and global ecosystem carbon budgets.

1

## 2 **Results**

3 In order to determine the amplitude and the steps of the leaf respiratory pathway that are  
4 inhibited in the light, detached leaves were fed with positionally labeled  $^{13}\text{C}$ -enriched  
5 substrates (Pyr or Glc) and decarboxylation rates were measured by gas-exchange coupled to  
6 isotopic spectrometry. This method allowed us to calculate the decarboxylation rate in the  
7 light and, by comparing with the rate in darkness, the inhibition of decarboxylation in  
8 illuminated leaves. The positional labeling in Pyr allowed us to discriminate between the  $\text{CO}_2$   
9 produced by either the PDH ( $^{13}\text{C}$ -1-Pyr,  $^{13}\text{C}$ -3-Glc) or the TCA cycle ( $^{13}\text{C}$ -2-Pyr,  $^{13}\text{C}$ -1-Glc).

10

### 11 ***TCA-mediated decarboxylations are enhanced under low $\text{CO}_2/\text{O}_2$***

12 When leaves were fed with  $^{13}\text{C}$ -enriched Pyr, the apparent carbon isotope discrimination  $\Delta_{obs}$   
13 increased, showing that  $^{13}\text{CO}_2$  was produced (Fig. 1A). Interestingly, the decarboxylation of  
14  $^{13}\text{C}$ -2-Pyr was low compared to that of  $^{13}\text{C}$ -1-Pyr, showing the predominance of  $\text{CO}_2$   
15 produced by PDH as opposed to that produced by the TCA cycle. In addition, the smaller the  
16  $\text{CO}_2/\text{O}_2$  ratio, the larger the  $\Delta_{obs}$  associated with  $^{13}\text{C}$ -1-Pyr. A similar but modest trend  
17 occurred with  $^{13}\text{C}$ -2-Pyr (Fig. 1A).

18 Dark-respired  $\text{CO}_2$  was  $^{13}\text{C}$ -enriched compared to the natural abundance ( $\delta^{13}\text{C}$  of  $-$   
19  $22.1 \pm 0.5\text{‰}$ ) with both  $^{13}\text{C}$ -1- and  $^{13}\text{C}$ -2-Pyr, indicating that decarboxylation of Pyr was  
20 substantial (Fig. 1B). The decarboxylation of  $^{13}\text{C}$ -1-Pyr in the dark was larger after a light  
21 period at low  $\text{CO}_2$  ( $140 \mu\text{L L}^{-1}$ ) compared to a high  $\text{CO}_2$ . Both  $^{13}\text{C}$ -1- and  $^{13}\text{C}$ -2-Pyr were  
22 more decarboxylated in the dark after leaves had been exposed to light under low- $\text{O}_2$   
23 conditions (Fig. 1B), and this was sensitive to  $\text{O}_2$ -conditions in the dark.

24 The isotopic data shown in Figure 1 were used to calculate the decarboxylation rates  
25 (Fig. 2) associated with PDH and the TCA cycle using mass-balance equations (see Materials

1 and Methods). While the decarboxylation rate associated with PDH was inhibited by only  
2 30% in the light (Fig. 2), TCA cycle-mediated decarboxylations were much lower in the light  
3 than in the dark, with an inhibition of around 80% under typical atmospheric conditions (400  
4  $\mu\text{L L}^{-1}$   $\text{CO}_2$  in 21%  $\text{O}_2$ ). The inhibition of the PDH-mediated decarboxylation was relatively  
5 constant under the  $\text{CO}_2$  and  $\text{O}_2$  conditions investigated. In contrast, TCA-mediated  
6 decarboxylations were much more sensitive to low  $\text{CO}_2$  conditions, with 35% inhibition only  
7 at 140  $\mu\text{L L}^{-1}$   $\text{CO}_2$  in 21%  $\text{O}_2$  (Fig. 2C). It should be noted that while the inhibition value  
8 associated with the TCA cycle was similar in 400  $\mu\text{L L}^{-1}$   $\text{CO}_2$  2%  $\text{O}_2$  and in 400  $\mu\text{L L}^{-1}$   $\text{CO}_2$   
9 21%  $\text{O}_2$ , the absolute decarboxylation value was larger in 2%  $\text{O}_2$  both in the dark and in the  
10 light (Fig. 2). This effect was due to a higher stomatal conductance in 2%  $\text{O}_2$  and  
11 subsequently, larger transpiration rates that induced a higher absorption of labeled compounds  
12 (data not shown). Such a positive effect of low  $\text{O}_2$  conditions on stomatal conductance has  
13 already been observed in *Xanthium strumarium* (15).

14

#### 15 ***The commitment to glycolysis is enhanced under low $\text{CO}_2/\text{O}_2$***

16 Similar experiments were carried out with  $^{13}\text{C}$ -enriched Glc to determine whether the  
17 glycolytic carbon flow changes under varying  $\text{CO}_2/\text{O}_2$  conditions. Under typical conditions  
18 (400  $\mu\text{L L}^{-1}$   $\text{CO}_2$  in 21%  $\text{O}_2$ ), leaves fed with  $^{13}\text{C}$ -1-Glc or  $^{13}\text{C}$ -3-Glc did not produce  
19 significant amounts of  $^{13}\text{CO}_2$  in the light, as indicated by the very small deviation of the  
20 apparent carbon isotope discrimination  $\Delta_{obs}$  (Table S1 of the Supporting Information). The  
21 same applied to high  $\text{CO}_2$ -to- $\text{O}_2$  conditions (1000  $\mu\text{L L}^{-1}$   $\text{CO}_2$  in 21%  $\text{O}_2$ ), with a  $\Delta_{obs}$  value of  
22  $24.5 \pm 0.5\%$ . In contrast, the decarboxylation of  $^{13}\text{C}$ -1-Glc and  $^{13}\text{C}$ -3-Glc became apparent in  
23 the light under low  $\text{CO}_2$  conditions (140  $\mu\text{L L}^{-1}$  in 21%  $\text{O}_2$ ), with  $\Delta_{obs}$  values increasing up to  
24  $106.4 \pm 13.8\%$  (with  $^{13}\text{C}$ -3-Glc, Table S1). This indicated that  $^{13}\text{C}$ -Glc could be oxidised in the  
25 light by glycolysis and that PDH and TCA activities were both at the origin of the respired

1  $^{13}\text{CO}_2$  from  $^{13}\text{C}$ -Glc. It should be noted that this increase was not an artefact caused by the  
2 low  $\text{CO}_2$  mole fraction in the chamber (making decarboxylated  $\text{CO}_2$  proportionally larger),  
3 because the  $\text{CO}_2$  mole fraction was taken into account in the mass-balance-based calculations.  
4 In darkness, the  $\delta^{13}\text{C}$  value of respired  $\text{CO}_2$  increased to 365‰ (with  $^{13}\text{C}$ -3-Glc) after  
5 photosynthesis at  $400\ \mu\text{L L}^{-1}\ \text{CO}_2$ , and 719‰ (with  $^{13}\text{C}$ -3-Glc) after photosynthesis at  $140\ \mu\text{L}$   
6  $\text{L}^{-1}\ \text{CO}_2$  in 21%  $\text{O}_2$  (Table S1). When these values were used to calculate decarboxylation  
7 values, it was found that both TCA- and PDH-mediated decarboxylations were inhibited by  
8 nearly 90% at  $400\ \mu\text{L L}^{-1}\ \text{CO}_2$ , and 60% at  $140\ \mu\text{L L}^{-1}\ \text{CO}_2$  in the light (Table S2 of the  
9 Supporting Information). These data show that leaf  $\text{CO}_2$  levels modulate the entry of Glc  
10 molecules into the glycolytic and respiratory pathways both in the light and the dark; Glc is a  
11 better respiratory substrate at low  $\text{CO}_2$  levels.

12

### 13 ***Distribution of the $^{13}\text{C}$ -label in metabolites***

14 To gain information on the changes in metabolic pathways under the different  $\text{CO}_2/\text{O}_2$   
15 conditions in the light, the fate of the  $^{13}\text{C}$ -atoms (from  $^{13}\text{C}$ -substrate feeding) was determined  
16 in leaf metabolites by  $^{13}\text{C}$ -NMR analyses. Leaves were fed with positionally-enriched (99%  
17  $^{13}\text{C}$ ) substrates under either 140, 400, or  $1000\ \mu\text{L L}^{-1}\ \text{CO}_2$  in 21%  $\text{O}_2$  and the positional  
18 isotopic abundances of identified metabolites (in % of  $^{13}\text{C}$ ), measured by NMR, are displayed  
19 as an isotopomics array (Fig. 3). As expected, hexoses and Glc- or Fru-moieties of Suc were  
20  $^{13}\text{C}$ -labeled when  $^{13}\text{C}$ -Glc was supplied to leaves so that several C-1 and C-6 positions formed  
21 clusters (these positions are redistributed by aldolase and triose-phosphates isomerase  
22 reactions). The C-4 and C-5 positions in hexoses clustered near to the C-3 positions,  
23 indicating that a redistribution of  $^{13}\text{C}$  label occurred in the light through the pentose-  
24 phosphate pathway.



1           The flux through the pentose-phosphate cycle may be estimated with both NMR and  
2 gas-exchange data. At  $140 \mu\text{L L}^{-1} \text{CO}_2$ , the  $^{13}\text{C}$ -amount in the C-atom positions redistributed  
3 by the pentose-phosphate cycle (i.e. the  $^{13}\text{C}$ -amount in C-2, C-3, C-4 and C-5 after  $^{13}\text{C}$ -1-Glc  
4 labeling and the  $^{13}\text{C}$ -amount in C-1, C-2, C-5 and C-6 after  $^{13}\text{C}$ -3-Glc labeling), as found by  
5 NMR after labeling, corresponds to a  $^{13}\text{C}$  flux of  $0.03 \mu\text{mol m}^{-2} \text{s}^{-1}$ . With the gas-exchange  
6 data, the flux through the pentose-phosphate cycle can be estimated by the excess of  
7 calculated  $\text{CO}_2$  production from the TCA with respect to the PDH, because the pentose-  
8 phosphate cycle involves the decarboxylation of the C-1 atom of Glc. With  $^{13}\text{C}$ -1-Glc  
9 labeling, The TCA-mediated decarboxylation rate was  $0.11 \mu\text{mol m}^{-2} \text{s}^{-1}$  while the PDH-  
10 mediated decarboxylation was  $0.07 \mu\text{mol m}^{-2} \text{s}^{-1}$  (Table S2 of the Supporting information).  
11 The  $\text{CO}_2$  production by the pentose-phosphate cycle was thus of  $0.04 \mu\text{mol m}^{-2} \text{s}^{-1}$ , a value  
12 that is very close to that obtained with NMR. Such a value is nevertheless not enough to  
13 explain the entire  $^{13}\text{CO}_2$  production from  $^{13}\text{C}$ -Glc at low  $\text{CO}_2$ , so that the enhancement of the  
14 commitment to glycolysis still holds under this condition.

15           The commitment to the TCA cycle can be assessed with  $^{13}\text{C}$ -labeling in organic acids.  
16 Some organic- and amino-acids clustered on the upper part of Fig. 3, with positional  $^{13}\text{C}$ -  
17 enrichment generally lower than 10%. However, the C-2 atom of malate and the C-2 and C-3  
18 atoms of Glu were clearly labeled when  $^{13}\text{C}$ -Pyr was supplied. This labeling was lower at high  
19  $\text{CO}_2$ , and the same applied to the C-2 and C-3 atoms of fumarate. Since these C-atom  
20 positions in malate, Glu and fumarate can only be labeled by the interplay (redistribution) of  
21 the TCA cycle, this labeling trend is indicative of an increase in TCA cycle activity. This  
22 view agrees with the increase of the  $^{13}\text{C}$ -abundance in Glu, succinate and fumarate from high  
23 to low  $\text{CO}_2/\text{O}_2$  ratios as shown in Fig. 4 (where  $^{13}\text{C}$ -abundances are relative to those found in  
24 malate in order to take into account variations in refixed decarboxylated  $^{13}\text{CO}_2$  by  
25 phosphoenolpyruvate carboxylase (PEPC) activity, as discussed below).

1            Nevertheless, the lower  $^{13}\text{C}$ -abundance in organic acids under high  $\text{CO}_2$  conditions  
2 may also partly be due to the diluting effect of assimilated  $^{12}\text{C}$ -enriched carbon (inlet  $\text{CO}_2$  has  
3 a  $\delta^{13}\text{C}$  value near to  $-50\text{‰}$ ). This effect is expected in malate and fumarate, which are two  
4 major metabolites accumulated by *Xanthium* leaves. There was indeed a significant and  
5 negative correlation between the isotopic enrichment in fumarate C-2/3 and malate C-2 and  
6 the quantity of these organic acids (Fig. 4, inset). However, the correlation was reversed in the  
7 case of Glu C-2 (Fig. 4, inset) while there was no correlation at all with other organic acids or  
8 amino-acids (data not shown). Therefore, we conclude that the lower  $^{13}\text{C}$ -enrichment in  
9 organic- and amino-acids at high  $\text{CO}_2$  conditions was not only caused by an isotopic dilution  
10 but also by the decrease of the commitment of  $^{13}\text{C}$ -Pyr to the TCA cycle.

11

## 12 ***$^{13}\text{CO}_2$ refixation***

13 While the trend of  $^{13}\text{C}$ -labeling in TCA cycle intermediates is clear, the rate of  
14 decarboxylation of  $^{13}\text{C}$ -enriched substrates in the light and the  $^{13}\text{C}$ -labeling of different  
15 metabolites may have been adulterated by  $^{13}\text{CO}_2$  refixation by either PEPC or Rubisco.  
16 Indeed,  $^{13}\text{CO}_2$  fixation by PEPC occurred under each  $\text{CO}_2$  condition investigated in the  
17 present study, as revealed by the  $^{13}\text{C}$ -enrichment in the C-4 of malate when  $^{13}\text{C}$ -1-Pyr or  $^{13}\text{C}$ -  
18 2-Pyr was supplied to leaves. However, the C-2 and C-3 positions in malate were also clearly  
19  $^{13}\text{C}$ -enriched under low  $\text{CO}_2$  conditions when fed with  $^{13}\text{C}$ -2-Pyr (Fig. 3), and this observation  
20 is consistent with an increased TCA cycle activity.

21            Metabolites could have been also  $^{13}\text{C}$ -labeled *via* photosynthetic  $^{13}\text{CO}_2$  refixation. The  
22 labeling of hexoses and Suc in the C-3 position after Pyr feeding (refixation of  $^{13}\text{CO}_2$   
23 decarboxylated from  $^{13}\text{C}$ -1-Pyr by Rubisco) did occur but the maximum positional  $^{13}\text{C}$ -  
24 abundance in C-3 indicates that the proportion of refixed  $^{13}\text{C}$  in the whole molecule was about  
25 0.7% only. Refixation of respired  $\text{CO}_2$  into starch was also assessed after gas-exchange and

1 on-line apparent  $\Delta_{obs}$  measurements (Fig. 1): the carbon isotope composition ( $\delta^{13}\text{C}$ ) of starch  
2 was between  $-36.5$  (minimum value obtained at  $1000 \mu\text{L L}^{-1} \text{CO}_2$ , with  $^{13}\text{C}$ -1-Pyr feeding)  
3 and  $-12.7\text{‰}$  (maximum value obtained at  $140 \mu\text{L L}^{-1} \text{CO}_2$  with  $^{13}\text{C}$ -1-Pyr feeding), indicating  
4 that the proportion of starch containing refixed  $\text{CO}_2$  was between 0 and 0.4% only (see Table  
5 S3 of the Supporting Information). Therefore, although Rubisco-mediated refixation of  
6 decarboxylated  $^{13}\text{CO}_2$  did occur, it was always very low.

7

### 8 ***Correlations with phosphorylated metabolites***

9 Phosphorylated metabolites, such as dihydroxyacetone phosphate (DHAP), are known to be  
10 important regulators of the primary carbon metabolism of plant leaves (12,16). Therefore, we  
11 measured the amounts of phosphorylated compounds by  $^{31}\text{P}$ -NMR spectroscopy on the same  
12 samples used for the  $^{13}\text{C}$ -labeling and  $^{13}\text{C}$ -NMR analyses. There was a clear linear and  
13 positive correlation between the activity of the TCA cycle, as witnessed by gas-exchange  
14 (values from Fig. 2), and the DHAP-to-Glc-6-phosphate ratio (Fig. 5, dashed line). In  
15 contrast, there was no statistically significant correlation with the DHAP/Pi ratio although a  
16 positive trend was apparent (Fig. 5, dotted line).

17

### 18 **Discussion**

19

20 Based on data from gas-exchange analyses to enzymatic activities (6,7,8,9), it has become  
21 widely accepted that leaf day respiration (non photorespiratory  $\text{CO}_2$  evolution in the light) is  
22 inhibited in the light (for a review, see 3). However, the rationale and the effect of  
23 environmental conditions on day respiration and its inhibition are uncertain and solving such  
24 an issue is critical to understand how leaves adapt carbon partitioning between export of  
25 photosynthates and respiration or N economy under varying natural conditions.  $\text{CO}_2$  and  $\text{O}_2$   
26 levels are two parameters of fundamental importance because the leaf internal  $\text{CO}_2/\text{O}_2$  ratio

1 changes when environmental conditions alter stomatal closure (e.g. drought). Here, we have  
2 developed isotopic methods to provide evidence that respiratory metabolism is up-regulated  
3 when the  $\text{CO}_2/\text{O}_2$  ratio decreases, and we argue that day respiration is an exquisite example of  
4 metabolic compromise between feedback inhibition by NADH and ATP, and 2-oxoglutarate  
5 precursor requirement for N metabolism.

6

### 7 **The regulation of the TCA cycle**

8 While no major effect on PDH-decarboxylation was observed, the carbon flow through the  
9 TCA cycle increased under low  $\text{CO}_2/\text{O}_2$  conditions, as evidenced by the larger  
10 decarboxylation rate of  $^{13}\text{C}$ -2-Pyr, so that the inhibition by light of the TCA cycle was 40% at  
11  $140 \mu\text{L L}^{-1} \text{CO}_2$  as compared to nearly 90% under typical conditions ( $400 \mu\text{L L}^{-1} \text{CO}_2$ , Figs. 1  
12 and 2). Furthermore, the  $^{13}\text{C}$ -labeling of succinate, citrate and Glu, as revealed by NMR  
13 tracing after  $^{13}\text{C}$ -2-Pyr feeding, increased as the  $\text{CO}_2$  mole fraction decreased (Fig. 4). In this  
14 context, the observed labeling in malate (Fig. 3), that indicated the simultaneous increase in  
15 PEPC activity, comes as no surprise: this is in agreement with the anapleurotic role of this  
16 enzyme, that compensates for 2-oxoglutarate consumption (for Glu synthesis) by feeding the  
17 TCA cycling with oxaloacetate molecules (17). The whole picture is thus consistent with an  
18 increased commitment to the TCA cycle and Glu production under low  $\text{CO}_2$  conditions.

19 The up-regulation of the TCA metabolism under low  $\text{CO}_2$  stems from a larger  
20 glycolytic carbon input, as evidenced by the enhancement of  $^{13}\text{C}$ -3-Glc decarboxylation (see  
21 the Result section and Table S1 and S2) and the slight  $^{13}\text{C}$ -labeling in malate C-2 after  $^{13}\text{C}$ -  
22 Glc feeding (Fig. 3). In addition, as a consequence of the lower photosynthetic  $\text{CO}_2$  fixation  
23 rate, the DHAP/Glc-6-P ratio decreased with  $\text{CO}_2$  mole fraction, and importantly, this ratio  
24 was strongly correlated with the inhibition of the TCA cycle (Fig. 5). The relative abundance  
25 of triose phosphates is already known to be a metabolic parameter that controls carbon entry

1 to glycolysis by the interplay of the effector Fru-2,6-bisphosphate (12), and promotes  
2 pyruvate kinase activity (18). It thus appears clear that it also controls the commitment of  
3 carbon molecules to the respiratory pathway. Uncertainty nevertheless remains about whether  
4 it acts indirectly on the TCA cycle (through the enhancement of glycolysis) or not.

5

## 6 **Interactions with photorespiration**

7 The higher carbon flow through the TCA cycle under low CO<sub>2</sub> may appear somewhat  
8 paradoxical as it has often been supposed that large photorespiratory rates inhibit  
9 mitochondrial respiratory enzymes (11,19), such as the NAD-dependent isocitrate  
10 dehydrogenase (11). Accordingly, Gly decarboxylase antisense lines of potato (*Solanum*  
11 *tuberosum*) have lower decarboxylation rates in the light (as revealed by <sup>14</sup>C-labeling  
12 experiments) and the ATP/ADP as well as the NADH/NAD<sup>+</sup> ratios are both higher than in the  
13 wild type (20). In addition, the predominance of NADH production by Gly oxidation over that  
14 by the TCA cycle has been shown using isolated mitochondria under ADP-limiting conditions  
15 (21). This might reduce the NAD<sup>+</sup> available for the mitochondrial dehydrogenase steps of the  
16 TCA cycle. Our results show that the inhibition of day respiration occurs whatever the  
17 CO<sub>2</sub>/O<sub>2</sub> ratio and furthermore, there is a lower inhibition value under very low-O<sub>2</sub> conditions  
18 (400 μL L<sup>-1</sup> CO<sub>2</sub>, 2% O<sub>2</sub>) as compared to high-CO<sub>2</sub> conditions (1000 μL L<sup>-1</sup> CO<sub>2</sub>, 21% O<sub>2</sub>)  
19 (Fig. 2). This would be consistent with a much reduced mitochondrial redox poise caused by  
20 the non-physiological, very low oxygen mole fraction.

21         However, we show here that the inhibition of the TCA cycle is relaxed under low-  
22 CO<sub>2</sub> conditions in 21% O<sub>2</sub> (see above). Isocitrate production is probably not influenced by  
23 such photorespiratory conditions, as citrate synthase remains active because of the very large  
24  $K_i(\text{ATP})$  (5 mmol L<sup>-1</sup>) and the absence of any NADH effect (22). As the mitochondrial NAD-  
25 dependent isocitrate dehydrogenase is believed to be inhibited in the light, we suggest that

1 isocitrate is processed by the cytosolic or mitochondrial NADP-dependent isocitrate  
2 dehydrogenase (23). This bypass would allow to sustain the necessary Glu flow under  
3 photorespiratory conditions.

4

#### 5 **Possible rationale**

6 The regulation of the TCA cycle by the CO<sub>2</sub>/O<sub>2</sub> conditions may be viewed as a side effect of  
7 the drop in the DHAP relative quantity on the commitment to glycolysis and therefore  
8 respiration (see above and Fig. 5). Unless there are other prevailing imperatives (such as the  
9 need of NADH to reduce photorespiration-derived hydroxypyruvate or H<sub>2</sub>O<sub>2</sub>), it also reflects  
10 an increased need for Glu to feed photorespiratory N-recycling when conditions shift to low  
11 CO<sub>2</sub> mole fractions, simply because the production of Gly from glycolate would require a  
12 higher Glu flow. Unsurprisingly then, the production of the Glu-precursor 2-oxoglutarate by  
13 the TCA cycle is enhanced. The argument that photorespiration is beneficial for Glu synthesis  
14 is in agreement with the positive correlation between photorespiration and leaf nitrate  
15 reduction (24,25). This scenario is also consistent with the results obtained in the cytoplasmic  
16 male sterile CMSII mutant of tobacco (affected in the mitochondrial respiratory complex I) in  
17 which the day respiratory rate is similar or even higher than that of the wild type while both  
18 photorespiration and N-metabolism (amino-acid synthesis) are enhanced (26,27).

19 Therefore, we argue that day respiratory homeostasis in leaves is likely to be the result  
20 of a compromise between two opposing forces: (i) an inhibition of respiration and glycolysis  
21 due to high mitochondrial NADH levels generated by photorespiration (Gly decarboxylation)  
22 and elevated ATP/ADP and DHAP levels generated by photosynthetic activity; (ii) a  
23 stimulation of the TCA cycle in order to adjust 2-oxoglutarate production to photorespiratory  
24 Glu demand. Such a compromise should be very dynamic, adjusting to changes in  
25 environmental conditions that modify stomatal closure, thereby altering leaf internal CO<sub>2</sub>/O<sub>2</sub>

1 balance. For example, water deficit, that leads to a low internal CO<sub>2</sub> mole fraction,  
2 presumably promotes day respiration and photorespiration. Therefore, in the summer months  
3 the quantitative significance of these metabolic changes should be evident in many C<sub>3</sub> crops  
4 and natural vegetation. However, it is probable that such a promoting effect may disappear  
5 on a long-term basis because of acclimation processes (28,29). Thus the extent to which the  
6 regulation of day respiration by CO<sub>2</sub> and O<sub>2</sub> conditions scale up to crop productivity and  
7 global carbon sequestration needs further experimental assessment.

8

9

## 10 **Material and methods**

### 11 ***Plant material***

12 Cocklebur (*Xanthium strumarium* L., Asteraceae) plants were grown in the greenhouse from  
13 seed in 100 mL pots of potting mix and transferred to 3 L pots after two weeks. Minimum  
14 photosynthetic photon flux density during a 16-h photoperiod was kept at approximately 400  
15  $\mu\text{mol m}^{-2} \text{s}^{-1}$  by supplementary lighting. Temperature and vapor pressure deficit were  
16 maintained at approximately 25.5/18.5°C and 1.4/1.2 kPa day/night, respectively. The carbon  
17 isotope composition ( $\delta^{13}\text{C}$ ) of CO<sub>2</sub> in the greenhouse air was  $-9.5 \pm 0.3\%$ . The third or fourth  
18 leaves (from the apical bud) were used for all measurements.

19

### 20 ***Gas exchange measurements***

#### 21 *a- Closed system (dark respiration)*

22 The respiration chamber was placed in a closed system, which was directly coupled to an  
23 elemental analyser (EA) NA-1500 (Carlo-Erba, Milan) through a 15-mL loop, as described in  
24 (30). After decarboxylating the system, respired CO<sub>2</sub> was accumulated until it reached nearly  
25 300  $\mu\text{L L}^{-1}$ . The loop was then shunted and the gas inside the loop was introduced into the EA

1 with helium for gas chromatography. The connection valve between the EA and the isotope  
2 ratio mass spectrometer (VG Optima, Micromass, Villeurbanne, France) was opened when  
3 the CO<sub>2</sub> peak emerged from the EA.

4

5 *b- Open system (photosynthesis and on-line carbon isotope discrimination)*

6 The photosynthesis system has already been described in (9). Briefly, a purpose-built  
7 assimilation chamber was connected in parallel to the sample air hose of the LI-6400 (Li-Cor  
8 Inc., Lincoln, NE). Leaf temperature was controlled at 21°C with circulating water from a  
9 cooling water bath to the jacket of the leaf chamber, and was measured with a copper-  
10 constantan thermocouple plugged to the thermocouple sensor connector of the LI-6400  
11 chamber/IRGA. Inlet air was adjusted to *ca.* 10 mmol mol<sup>-1</sup> H<sub>2</sub>O and passed through the  
12 chamber at a rate of 30 L h<sup>-1</sup>, monitored by the LI-6400. Light (400 μmol m<sup>-2</sup> s<sup>-1</sup>) was  
13 supplied by a 500 W halogen lamp (Massive N. V., Kontich, Belgium). Inlet CO<sub>2</sub> was  
14 obtained from a gas cylinder (Alphagaz N48, Air Liquide, France) with a δ<sup>13</sup>C of –  
15 50.2±0.2‰. The outlet air of the chamber was regularly shunted and was sent to the loop to  
16 measure its <sup>12</sup>C/<sup>13</sup>C isotope composition and thus the on-line carbon isotopic discrimination  
17 (Δ<sub>obs</sub>). The gas inside the loop was introduced into the EA for GC as described above. Δ<sub>obs</sub>  
18 during photosynthesis was measured following the method described by ref. (31). Air with  
19 2% oxygen was from a cylinder (Crystal gas mixture, Air Liquide, France). When light was  
20 turned off, the leaf was immediately removed from the open system and one half was frozen  
21 in liquid nitrogen. The other half (still attached to the peduncle) was placed in the closed  
22 system for dark respiration measurements (see above).

23



1 ***Starch extraction***

2 The protocol for starch extraction was similar to that described in (30). The frozen leaf  
3 material was lyophilised and powdered. 50 mg of leaf powder was suspended with 1 mL of  
4 distilled water in an Eppendorf tube (Eppendorf Scientific, Hamburg, Germany). After  
5 centrifugation, the pellet was washed four times with 95% ethanol at room temperature and  
6 starch was extracted by HCl solubilization and precipitated with cold methanol. After  
7 lyophilisation, starch was transferred to tin capsules (Courtage Analyze Service, Mont Saint-  
8 Aignan, France) for isotope analysis.

9

10 ***NMR analyses***

11 Leaves used for NMR spectroscopy were fed for 2 h at 21°C and 400  $\mu\text{mol m}^{-2} \text{s}^{-1}$  with either  
12 water (control),  $^{12}\text{C}$ -substrates or  $^{13}\text{C}$ -substrates in a large plexiglass chamber (surface area  
13 450  $\text{cm}^2$ ) connected in parallel to the sample air hose of the LI-6400 (Li-Cor Inc., Lincoln,  
14 NE), allowing  $\text{CO}_2$  mole fraction monitoring.

15 NMR measurements were carried out as described in (9) and (32) from perchloric acid  
16 extracts prepared from 5 g of frozen leaf material. Spectra were obtained using a Bruker  
17 spectrometer (AMX 400) equipped with a 10-mm multinuclear probe tuned at 161.9 and  
18 100.6 MHz for  $^{31}\text{P}$ - and  $^{13}\text{C}$ -NMR, respectively. The assignment of  $^{13}\text{C}$  resonance peaks was  
19 carried out according to (33). Identified compounds were quantified from the area of their  
20 resonance peaks using fully relaxed conditions for spectra acquisition (pulses at 20 s  
21 intervals). Peak intensities were normalized to a known amount of the reference compound  
22 (maleate for  $^{13}\text{C}$  and methyl-phosphonate for  $^{31}\text{P}$ ) that was added to the sample (internal  
23 standard).

24

25

## 1 <sup>13</sup>C-enriched molecules

2 The positional <sup>13</sup>C-labeled molecules (99% <sup>13</sup>C in the considered position) were purchased  
3 from Eurisotop (Saclay, France). Pyruvate was dissolved in distilled water and the pH was  
4 adjusted to 6.7 with NaOH. To obtain non-fully labeled solutions ( $\Delta_{obs}$  experiments), the  
5 labeled compounds were mixed with industrial glucose ( $\delta^{13}\text{C} = -9\text{‰}$ ) or pyruvate  
6 ( $\delta^{13}\text{C} = -21\text{‰}$ ) from Sigma. The resulting overall composition of glucose and pyruvate  
7 solutions was checked to be 2500‰ and 1400‰, respectively. In each case, the final  
8 concentration was 0.015 mol L<sup>-1</sup>. The solutions were fed to the leaves through the  
9 transpiration stream.

10

11

12

## 13 **Calculations**

14 The procedure used to calculate the decarboxylation rates of <sup>13</sup>C-enriched substrates in the  
15 light from apparent  $\Delta_{obs}$  values has already been explained in detail (9). Briefly, the difference  
16 between apparent  $\Delta_{obs}$  values obtained with and without substrate addition is considered to  
17 reflect the additional decarboxylation flux in the light. Using mass balance equations, it can  
18 be shown that the decarboxylation rate  $r_{day}$  has the following form:

19 
$$r_{day} = \frac{d}{SV_M} \cdot \frac{c_o \lambda_o - c_e \lambda_e + (c_e - c_o) \lambda_{fixed}}{\lambda_s - \lambda_{fixed}}$$

20 where  $d$  is the flow,  $S$  leaf surface area,  $V_M$  the molar volume at air temperature,  $c_e$  and  $c_o$  are  
21 the CO<sub>2</sub> mole fractions in inlet and outlet air, respectively.  $\lambda$  values are <sup>13</sup>C percentages (using  
22 delta-values is not possible because of large <sup>13</sup>C-enrichments) in inlet CO<sub>2</sub> (subscript  $e$ ), outlet  
23 CO<sub>2</sub> (subscript  $o$ ), net fixed CO<sub>2</sub> (subscript  $fixed$ ), and <sup>13</sup>C-enriched added substrate (subscript  
24  $s$ ). This equation holds for homogeneously labeled substrates; it is somewhat changed for

1 positional enrichments to take into account the different origin of decarboxylated CO<sub>2</sub>. This  
 2 occurs when glucose or pyruvate are added: the C-1 atom of pyruvate is decarboxylated by  
 3 PDH while the C-2 and C-3 positions are decarboxylated by the Krebs cycle. Similarly, the C-  
 4 3 and C-4 atom positions of glucose are decarboxylated by the PDH reaction, the other being  
 5 decarboxylated by the Krebs cycle. Taking advantage of positionally <sup>13</sup>C-enriched substrates:  
 6 <sup>13</sup>C-1-pyruvate would specifically enrich the CO<sub>2</sub> produced by PDH while <sup>13</sup>C-2-pyruvate  
 7 would specifically enrich the CO<sub>2</sub> that comes from the Krebs cycle. The same applies to  
 8 positional <sup>13</sup>C-enrichment in glucose. It should be noted that, in contrast to the argument of  
 9 ref. (3), any isotopic dilution of the substrate is taken into account as the observed carbon  
 10 isotope discrimination is always a net value that integrates the decarboxylation of natural (that  
 11 is, not added) Pyr or Glc molecules, both before and after <sup>13</sup>C-substrate addition.

12 A similar procedure applies to dark-respired CO<sub>2</sub> measurements. In other words, CO<sub>2</sub>  
 13 that is produced in darkness (<sup>13</sup>C-percentage  $\lambda_{global}$ ) after a light period with <sup>13</sup>C-enriched  
 14 substrate feeding comes from respiratory oxidation of new photosynthates (the <sup>13</sup>C-percentage  
 15 in the net fixed carbon is  $\lambda_{fixed}$ ), photosynthates from the previous light period in the  
 16 greenhouse (<sup>13</sup>C-percentage  $\lambda_{previous}$ ), and additional C coming from the <sup>13</sup>C-enriched substrate  
 17 fed to the leaf (<sup>13</sup>C percentage  $\lambda_s$ ). The night-decarboxylation rate has the following form:

$$18 \quad r_{night} = R_n \cdot \frac{\lambda_{global} - \lambda_p}{\lambda_s - \lambda_p}$$

19 where  $\lambda_p$  is a linear combination of  $\lambda_{previous}$  and  $\lambda_{fixed}$ . It is equal to 0.6  $\lambda_{previous}$  + 0.4  $\lambda_{fixed}$  after  
 20 2-3 h in the light under ordinary CO<sub>2</sub>/O<sub>2</sub> conditions (during which 200-400 mmol C m<sup>-2</sup> have  
 21 been fixed), and 0.5  $\lambda_{previous}$  + 0.5  $\lambda_{fixed}$  after 2-3 h in the light under high CO<sub>2</sub> conditions  
 22 (during which 400-800 mmol C m<sup>-2</sup> have been fixed) (34). It should be noted that possible  
 23 variations in these coefficients do only introduce very slight errors in the estimate of the <sup>13</sup>C-  
 24 enriched substrate decarboxylation  $r_{night}$ , because of the strong <sup>13</sup>C-enrichment of the

1 substrate (that is, the  $\lambda_p$  value is always very small compared to  $\lambda_{global}$  or  $\lambda_s$  and may be  
2 neglected). Again, that relationship is somewhat modified with positional enrichments to take  
3 into account the different origin of decarboxylated CO<sub>2</sub> (as described in 9).

4

#### 5 ***Clustering analysis***

6 The <sup>13</sup>C-NMR data were represented as an isotopic array as described in (35). The  
7 positional isotopic abundances (in <sup>13</sup>C-percentage) relative to the natural <sup>13</sup>C-abundance  
8 (1.1%) are indicated by colors so that black cells indicate near-natural abundance, green and  
9 red cells indicate lower- and larger-than-natural <sup>13</sup>C-abundance. The clustering analysis was  
10 carried out with the Cluster software and the array is drawn using the TreeView software  
11 (both are from M. Eisen, Stanford University).

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**Figure 1.** **A**, carbon isotope discrimination ( $\Delta_{obs}$ ) associated with photosynthesis of detached leaves (at 21°C and 400  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD) fed with either  $^{13}\text{C}$ -1- or  $^{13}\text{C}$ -2-enriched Pyr under four  $\text{CO}_2/\text{O}_2$  conditions: 140, 400 or 1000  $\mu\text{L L}^{-1}$   $\text{CO}_2$  in 21%  $\text{O}_2$ , and 400  $\mu\text{L L}^{-1}$   $\text{CO}_2$  in 2%  $\text{O}_2$ . **B**, carbon isotope composition ( $\delta^{13}\text{C}$ ) of respired  $\text{CO}_2$  in darkness after the corresponding light periods. When leaves experienced a light period under 2%  $\text{O}_2$ , the carbon isotope composition was measured in either 21% (indicated as '400  $\mu\text{L L}^{-1}$ , 2-21%') or 2%  $\text{O}_2$  (indicated as '400  $\mu\text{L L}^{-1}$ , 2-2%'). Each value is the mean $\pm$ SE of three measurements. The control  $\delta^{13}\text{C}$  value of respired  $\text{CO}_2$  was  $-22.1\pm 0.5\%$ .

**Figure 2.** Decarboxylation rates and inhibition of decarboxylation by light calculated from data of Fig. 1, using the method of ref. (9). The decarboxylation rates by PDH (white bars) and the TCA cycle (black bars) are given in the light (panel **A**, denoted as  $r_{light}$  just below) and in the dark (panel **B**, denoted as  $r_{night}$  just below). Inhibition by light (calculated as  $1 - r_{light}/r_{night}$ , in %) is indicated on panel **C**. Conditions experienced by leaves during the light period are indicated on the  $x$  axis as in Fig. 1: 140, 400 and 1000  $\mu\text{L L}^{-1}$   $\text{CO}_2$  in 21%  $\text{O}_2$  and 400  $\mu\text{L L}^{-1}$   $\text{CO}_2$  in 2%  $\text{O}_2$ . The  $\delta^{13}\text{C}$  values of dark-respired  $\text{CO}_2$  obtained in 2%  $\text{O}_2$  (Fig. 1B right bars) were used to calculate the inhibition value after a light period in 2%  $\text{O}_2$ .

**Figure 3.** Isotopomics array representation of  $^{13}\text{C}$ -abundance in the carbon atom positions of major metabolites in detached leaves incubated with  $^{13}\text{C}$ -substrates for 2 h at 21°C, 21%  $\text{O}_2$  and 400  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD.  $\text{CO}_2$  mole fraction was 140  $\mu\text{L L}^{-1}$ , 400  $\mu\text{L L}^{-1}$ , and 1000  $\mu\text{L L}^{-1}$ . At  $t = 2$  h, leaves were immediately frozen in liquid nitrogen for perchloric acid extraction. Perchloric extracts were analysed for positional  $^{13}\text{C}$ -abundances by NMR. Each column is a separate set of experimental conditions. Cit, citrate; Fum, fumarate; Ido/Gal, uncertain D-hexofuranose belonging to the idose-galactose group; Mal, malate; Obt, oxobutyrate; SF and

SG, fructosyl and glucosyl moieties of sucrose, respectively. Red and green cells indicate  $^{13}\text{C}$ -abundances above and below the natural abundance (which is 1.1%). Below-natural abundance cells appear dark-green because the  $^{13}\text{C}$ -abundance is still very close to 1.1%.

**Figure 4. Main panel.**  $^{13}\text{C}$ -abundance in glutamate (Glu, black bars), succinate (Succ, light-grey bars) and fumarate (Fum, dark-grey bars), relative to that in malate. Values are from the data of Fig. 3. The three different  $\text{CO}_2$  mole fractions (in  $\mu\text{mol mol}^{-1}$ ) used in the experiment are indicated on the  $x$  axis. **Inset.** Data of Fig. 3, replotted to show the relationship between the positional  $^{13}\text{C}$ -abundance (in % of  $^{13}\text{C}$ ) in malate C-2 (triangles), fumarate C-2/3 (circles) and glutamate C-2 (squares) and the quantity of metabolite (in  $\mu\text{mol per gram of fresh weight}$ ). Short dashed lines indicate exponential decay (fumarate and malate) and linear (Glu) regressions; both are significant:  $F=7.26$  ( $P<0.005$ ) and  $F=16.38$  ( $P<0.003$ ), respectively.

**Figure 5.** Relationship between the inhibition of the TCA cycle in the light (in %, data from Fig. 2) and the dihydroxyacetone phosphate (DHAP) to inorganic phosphate (Pi) (open discs) or Glc-6-phosphate (closed disks) ratio. Phosphorylated compounds were measured by  $^{31}\text{P}$ -NMR on the same samples used for  $^{13}\text{C}$ -NMR after  $^{13}\text{C}$ -labeling. Lines stand for linear regressions. The regression with DHAP/Glc-6-P is significant ( $F=61.7$ ,  $P<0.08$ ).



Figure 1

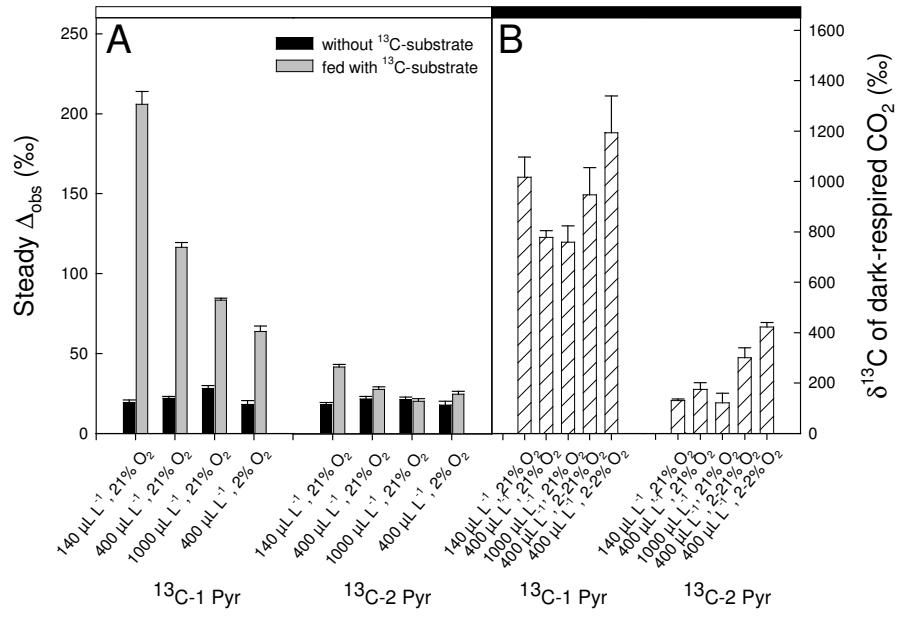


Figure 2

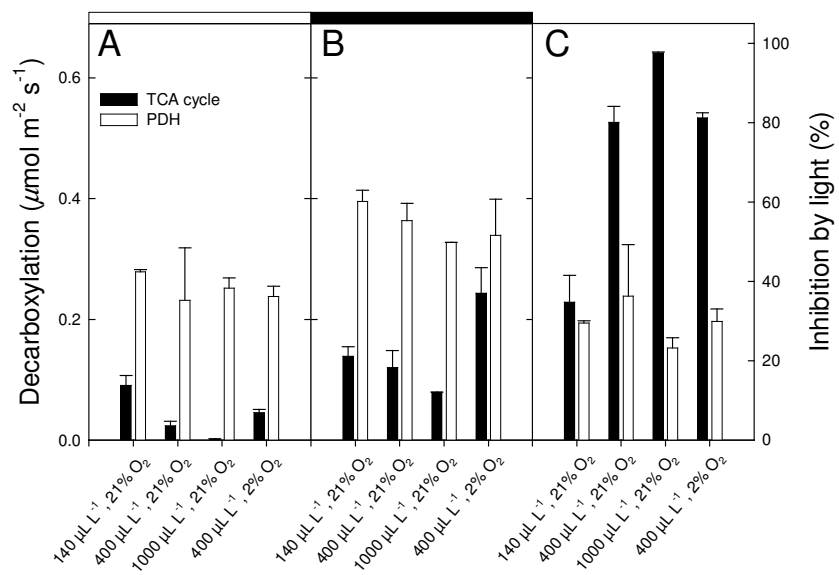


Figure 3

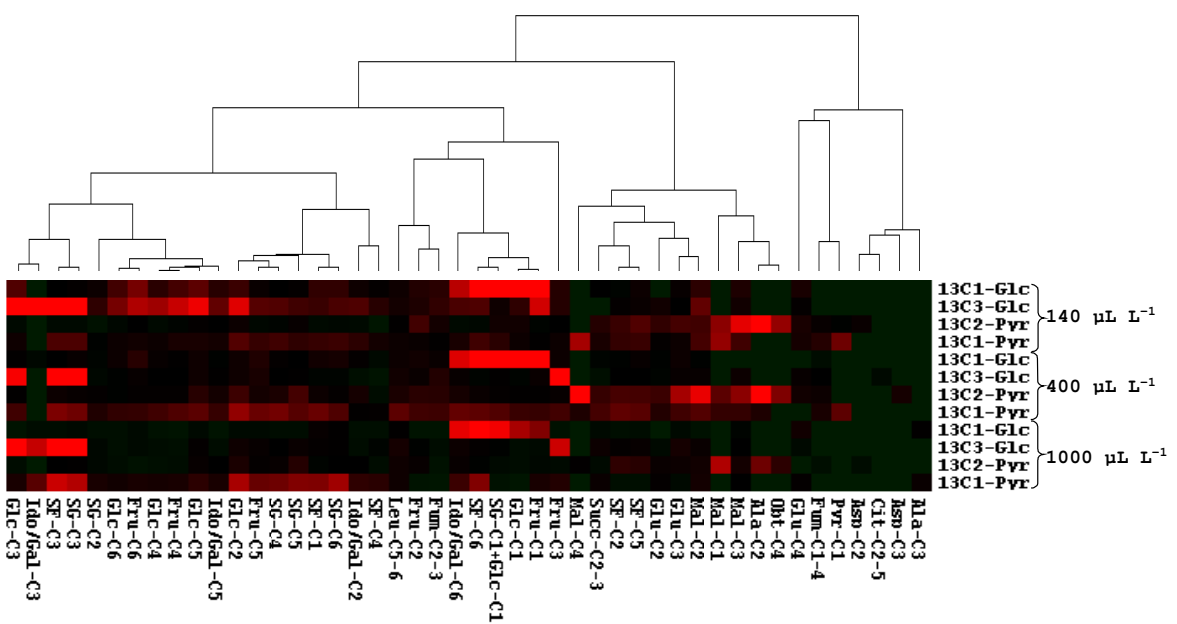


Figure 4

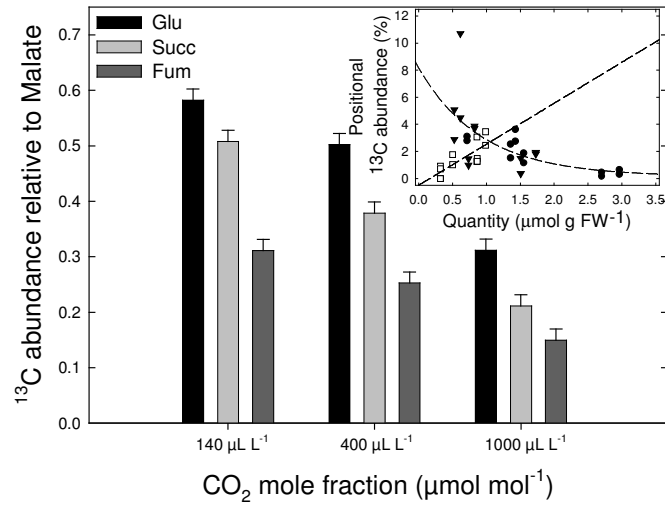
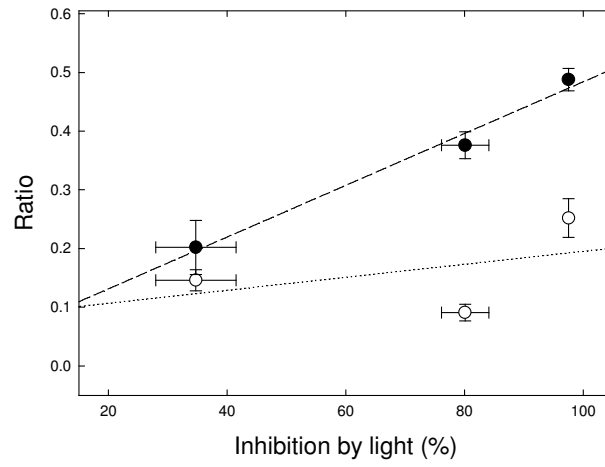


Figure 5



### Supporting information (for on-line publication)

**Table S1.** Apparent photosynthetic carbon isotope discrimination ( $\Delta_{\text{obs}}$ ) and carbon isotope composition of dark-respired  $\text{CO}_2$  ( $\delta^{13}\text{C}$ ) of detached leaves fed with  $^{13}\text{C}$ -1- or  $^{13}\text{C}$ -3-Glc. Conditions during the light period are 140 and 400  $\mu\text{L L}^{-1}$   $\text{CO}_2$  in 21%  $\text{O}_2$  under 400  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD. In water (no feeding conditions), the  $\Delta_{\text{obs}}$  value is  $21.6 \pm 1.2\text{‰}$ . The  $\delta^{13}\text{C}$  value of respired  $\text{CO}_2$  in darkness in water (no feeding) is  $-34.5 \pm 0.8\text{‰}$  and  $-42.0 \pm 0.7\text{‰}$  after a light period under 140  $\mu\text{L L}^{-1}$  and 400  $\mu\text{L L}^{-1}$   $\text{CO}_2$ , respectively. Mean and SE of three measurements.

140 $\mu\text{L L}^{-1}$ $\text{CO}_2$ in 21% $\text{O}_2$				400 $\mu\text{L L}^{-1}$ $\text{CO}_2$ in 21% $\text{O}_2$			
$^{13}\text{C}$ -1-Glc		$^{13}\text{C}$ -3-Glc		$^{13}\text{C}$ -1-Glc		$^{13}\text{C}$ -3-Glc	
Light	Darkness	Light	Darkness	Light	Darkness	Light	Darkness
$\Delta_{\text{obs}}$ , ‰	$\delta^{13}\text{C}$ , ‰	$\Delta_{\text{obs}}$ , ‰	$\delta^{13}\text{C}$ , ‰	$\Delta_{\text{obs}}$ , ‰	$\delta^{13}\text{C}$ , ‰	$\Delta_{\text{obs}}$ , ‰	$\delta^{13}\text{C}$ , ‰
56.0±5.6	386±8	106.4±13.8	719±30	23.8±1.0	245±8	23.8±1.0	365±33

**Table S2.** Glucose decarboxylation rates and inhibition of decarboxylation by light, using the method of ref. (9) and data from Table S1. The decarboxylation rates by PDH and the TCA cycle are given in the light (denoted as  $r_{\text{light}}$  just below) and in the dark (denoted as  $r_{\text{night}}$  just below). Inhibition by light (calculated as  $1 - r_{\text{light}}/r_{\text{night}}$ , in %) is indicated on the right.

	140 $\mu\text{L L}^{-1}$ $\text{CO}_2$ in 21% $\text{O}_2$		400 $\mu\text{L L}^{-1}$ $\text{CO}_2$ in 21% $\text{O}_2$	
	PDH decarboxylation	TCA decarboxylation	PDH decarboxylation	TCA decarboxylation
Decarboxylation value in the light, $\mu\text{mol m}^{-2} \text{s}^{-1}$	0.067±0.011	0.111±0.001	0.007±0.001	0.019±0.001
Decarboxylation value in darkness, $\mu\text{mol m}^{-2} \text{s}^{-1}$	0.205±0.017	0.267±0.001	0.074±0.001	0.204±0.001
Inhibition by light, %	67.4±2.8	58.5±0.1	89.4±0.7	90.7±0.1

**Table S3.** Carbon isotope composition of starch (in ‰) after feeding leaves with <sup>13</sup>C-enriched Glc (δ<sup>13</sup>C=2500‰) or Pyr (δ<sup>13</sup>C=1400‰) in the light under different CO<sub>2</sub>/O<sub>2</sub> conditions (experiment described in Figure 1) and associated contributions of <sup>13</sup>C-enriched substrates to starch synthesis. The δ<sup>13</sup>C value of CO<sub>2</sub> used in the open system is -50.2‰. Samples were immediately frozen in liquid nitrogen after 2 hours feeding, for starch extraction. The contribution values assume refixation of the <sup>13</sup>C-enriched decarboxylated CO<sub>2</sub> and do not take into account the global δ<sup>13</sup>C of added substrate. Contributions given in μmol m<sup>-2</sup> s<sup>-1</sup> are average values over the 2 h feeding period. Mean and SD of three measurements.

	140 μL L <sup>-1</sup> CO <sub>2</sub> in 21% O <sub>2</sub>				400 μL L <sup>-1</sup> CO <sub>2</sub> in 21% O <sub>2</sub>				1000 μmol mol <sup>-1</sup> CO <sub>2</sub> in 21% O <sub>2</sub>	400 μmol mol <sup>-1</sup> CO <sub>2</sub> in 2% O <sub>2</sub>		
	<sup>13</sup> C-1-Pyr	<sup>13</sup> C-2-Pyr	<sup>13</sup> C-1-Glc	<sup>13</sup> C-3-Glc	<sup>13</sup> C-1-Pyr	<sup>13</sup> C-2-Pyr	<sup>13</sup> C-1-Glc	<sup>13</sup> C-3-Glc	<sup>13</sup> C-1-Pyr	<sup>13</sup> C-2-Pyr	<sup>13</sup> C-1-Pyr	<sup>13</sup> C-2-Pyr
No feeding	-28.0±1.0				-31.4±0.4				-35.4±0.9		-33.0±0.9	
Fed leaf	-12.7±0.5	-27.4±0.4	-18.3±0.5	-13.3±0.6	-21.8±1.0	-29.9±0.7	-27.3±0.6	-27.7±0.2	-36.5±0.5	-32.5±0.4	-22.3±0.3	-27.4±0.7
Contribution (%)	0.37	0.01	0.13	0.20	0.23	0.03	0.06	0.05	0	0.06	0.26	0.13
Contribution (μmol m <sup>-2</sup> s <sup>-1</sup> )	0.06	0	0.02	0.03	0.04	0.01	0.01	0.01	0	0.01	0.04	0.02