In Vivo Respiratory Metabolism of Illuminated Leaves[1]

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Day respiration of illuminated C_3 leaves is not well understood and particularly, the metabolic origin of the day respiratory CO_2 production is poorly known. This issue was addressed in leaves of French bean (*Phaseolus vulgaris*) using $^{12}C/^{13}C$ stable isotope techniques on illuminated leaves fed with ^{13}C-enriched glucose or pyruvate. The $^{13}CO_2$ production in light was measured using the deviation of the photosynthetic carbon isotope discrimination induced by the decarboxylation of the ^{13}C-enriched compounds. Using different positional ^{13}C-enrichments, it is shown that the Krebs cycle is reduced by 95% in the light and that the pyruvate dehydrogenase reaction is much less reduced, by 27% or less. Glucose molecules are scarcely metabolized to liberate CO_2 in the light, simply suggesting that they can rarely enter glycolysis. Nuclear magnetic resonance analysis confirmed this view; when leaves are fed with ^{13}C-glucose, leaf sucrose and glucose represent nearly 90% of the leaf ^{13}C content, demonstrating that glucose is mainly directed to sucrose synthesis. Taken together, these data indicate that several metabolic down-regulations (glycolysis, Krebs cycle) accompany the light/dark transition and emphasize the decrease of the Krebs cycle decarboxylations as a metabolic basis of the light-dependent inhibition of mitochondrial respiration.

Illuminated leaves simultaneously assimilate CO_2 through the photosynthetic carbon reduction cycle and lose CO_2 through photorespiration and day respiration. In darkness, leaves no longer assimilate CO_2 via the photosynthetic carbon reduction cycle but produce CO_2 through dark respiration. Although dark respiration is known to involve glycolysis and CO_2 production through pyruvate dehydrogenation and the degradative Krebs cycle (Trethewey and ap Rees, 1994; Plaxton, 1996), the carbon metabolism that is responsible for the CO_2 respiratory release in the light is almost unknown. This is so because the day respiratory CO_2 flux is very low and masked by the photosynthetic carbon fixation and the photorespiratory CO_2 production in the light, and is thus difficult to study.

Nevertheless, it has been repeatedly shown, using either the Laisk's (Laisk, 1977) or Kok's method (Kok, 1948), that the rate of day respiration (R_d) is less than that of dark respiration (R_n; for review, see Atkin et al., 2000) so that light is known to inhibit respiration, with a R_d/R_n value (usually denoted as μ) ranging from 30% to 100% (for a recent study, see Peisker and Apel, 2001). Pioneering gas exchange measurements on mustard suggested that some enzymatic activities are inhibited in the light so that substrates accumulate (Cornic, 1973), explaining the respiratory burst when leaves are darkened: the light enhanced dark respiration. More recently, it has been shown in the unicellular alga *Selenastrum minutum* that pyruvate kinase (Lin et al., 1989) is inhibited by light. It is also the case of the pyruvate dehydrogenase complex that is partly inactivated by (reversible) phosphorylation in extracts from illuminated leaves (Budde and Randall, 1990; Tovar-Mendez et al., 2003). Photorespiration is also probably involved in the inhibition of pyruvate dehydrogenase as it has been shown that this enzyme is down-regulated by NH_3, which is a byproduct of the photorespiratory Gly decarboxylation (Krömer, 1995). Enzymes of the Krebs cycle are also assumed to be inhibited in the light because of a high mitochondrial NADH level due to photorespiratory Gly decarboxylation (Atkin et al., 2000). Additionally, it has been shown that the mitochondrial isocitrate dehydrogenase is inhibited by the high NADPH/NADP ratios that occur in the light (Igamberdiev and Gardeström, 2003).

Although all these enzymatic data suggest that the respiratory pathway is down-regulated in the light regarding both glycolysis and the Krebs cycle, respiratory metabolic fluxes in vivo in leaves are not well known. Some labeling experiments with carbon isotopes (^{13}C or ^{14}C) have already been done to disentangle respiratory metabolic fluxes in vivo in the light and in the dark, but surprisingly, studies that have focused on labeling of the resulting respired CO_2 are scarce. Using $^{14}CO_2$ labeling techniques, Pärnik et al. (2002) suggested that CO_2 production in the light is composed of (1) decarboxylation of primary products like triose phosphates and malate (between 10% and 50%

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from one species to another), and (2) decarboxylation of end-products like Suc and starch to a greater extent (50%–90%). $^{12}CO_2$ (respiratory) production in a ^{13}C atmosphere has been used to show that day respiration is less than night respiration, and its rate as well as the ratio μ decreased at high CO_2 concentration (Pinelli and Loreto, 2003). It has thus been proposed that inhibition of CO_2 production is determined by the CO_2 fixation flux (Atkin et al., 1998; Pinelli and Loreto, 2003). The ability of leaves to oxidize some metabolites through respiration has also been investigated with feeding experiments using labeled organic compounds. Supplying ^{13}C-enriched Glc to myrtle leaves in the light with CO_2-free air did enrich the CO_2 subsequently respired in the dark, although the CO_2 was not completely labeled (Affek and Yakir, 2003). Nevertheless, the ^{13}C amount in the light-respired CO_2 was not measured in that study.

So already published data do not show what the respiratory metabolic pathway is actually in illuminated leaves; that is, what the metabolic fluxes associated with the day respiratory CO_2 production are. We address this question here by feeding illuminated French bean (*Phaseolus vulgaris*) leaves with positional ^{13}C-enriched Glc and pyruvate and measuring the resulting ^{13}C-enrichment in both day and dark-respired CO_2 and in intermediary respiratory compounds, using isotope ratio mass spectrometry and nuclear magnetic resonance (NMR), respectively. The isotopic analysis of CO_2 respired in the dark and the measurement of the carbon isotope discrimination during photosynthesis allows us to calculate the ^{13}C-content in dark- and light-respired CO_2, respectively. Respiration of supplied $^{13}C_1$- and $^{13}C_3$-enriched Glc is found to be almost completely inhibited by light. By contrast, decarboxylation of $^{13}C_1$-enriched pyruvate through the pyruvate decarboxylation is much less inhibited by light, unlike ^{13}C-2-pyruvate. These data are supported by NMR spectra obtained from illuminated leaves and taken as a whole, they suggest that (1) the Krebs cycle and glycolysis are strongly inhibited by light, with little interconversion between triose phosphates and hexose phosphates through the triose phosphate isomerase and aldolase reactions, and (2) the pyruvate dehydrogenation is only partly inhibited by light, with the acetyl-CoA molecules being directed toward purposes other than respiration.

THEORY

This "Theory" section describes the mathematical background used to calculate the R_d decarboxylations when detached leaves were supplied with ^{13}C-enriched molecules (Fig. 1).

In the following, the isotope composition ($\delta^{13}C$) and the isotope ratio ($^{13}C/^{12}C$) are denoted as δ and R, respectively, and the percentage of ^{13}C is denoted as λ. λ is simply deduced from δ through the following relationship:

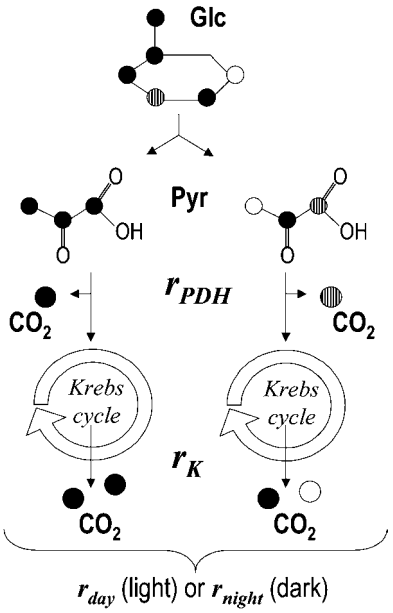

Figure 1. Metabolic model used in this paper for decarboxylations of ^{13}C-enriched substrates and respiratory variables taken into account (r_{PDH} and r_K and the sum, r_{day} or r_{night}). r_{PDH} and r_K are the rates of decarboxylation of ^{13}C-enriched substrates through the PDH reaction and the Krebs cycle, respectively. r_{day} and r_{night} are the sum of r_{PDH} and r_K in the light and in the dark, respectively. Different symbols are used for different carbon atom positions in order to see the pathways to CO_2 production.

$$\lambda = \frac{^{13}C}{^{13}C + ^{12}C} = \frac{^{13}C/^{12}C}{^{13}C/^{12}C + 1} = \frac{R}{R+1}.$$

As $\delta = \frac{R - R_{st}}{R_{st}}$ where R_{st} is the $^{13}C/^{12}C$ ratio in the Pee Dee reference material ($R_{st} = 0.0112372$), we have:

$$\lambda = \frac{1}{1 + \frac{1}{R_{st}(\delta + 1)}}. \quad (1)$$

Rate of ^{13}C-Enriched Substrate Decarboxylation in the Light (r_{day})

The on-line discrimination value was obtained using the method of Evans et al. (1986):

$$\Delta_{obs} = \frac{\xi(\delta_e - \delta_o)}{1 + \delta_o - \xi(\delta_e - \delta_o)},$$

where the isotope compositions of air entering and leaving the cuvette are δ_e and δ_o, respectively. ξ is equal to $c_e/(c_e - c_o)$ where c_e and c_o are the CO_2 molar fractions in the entering and leaving air, respectively.

The Δ_{obs} value obtained before the substrate addition is denoted as Δ. It is assumed that after substrate addition, the air leaving the leaf cuvette is the sum of the CO_2 left by photosynthetic discrimination and additional CO_2 released from substrate respiration. The latter flux is denoted as r_{day}. If the leaf area is denoted as S and c_{fixed} is the CO_2 amount (in μL/L)

fixed by photosynthesis, the mass balance equation for CO_2 is as follows:

$$c_e + \frac{r_{day}}{d} SV_M = c_{fixed} + c_o,$$

where d (L/s) is the air flow through the leaf cuvette and V_M the molar volume. Thus, we have:

$$c_{fixed} = c_e + \frac{r_{day}}{d} SV_M - c_o. \quad (2)$$

Case of Feeding with a Compound That Has a Homogeneous Isotope Composition

Homogeneous isotopic compounds are not involved in this study, but the calculation is explained here as it gives a basis to understand the next step, that is, the use of compounds with a heterogeneous isotope composition.

The isotope composition of the substrate fed to the leaf is denoted as δ_s. Isotopic mass balance is so that the ^{13}C amount entering the cuvette ($c_e\lambda_e$) plus that from the additional CO_2 release ($r_{day}SV_M\lambda_s/d$) is equal to the sum of the ^{13}C amount fixed by the leaf ($c_{fixed}\lambda_{fixed}$) and the ^{13}C amount leaving the cuvette ($c_o\lambda_o$). That is,

$$c_e\lambda_e + \frac{r_{day}}{d} SV_M\lambda_s = c_{fixed}\lambda_{fixed} + c_o\lambda_o. \quad (3)$$

Note that the similar relationship with deltas is not correct as the strong ^{13}C-enrichment does not allow one to neglect R compared to 1 so that $\lambda \neq R$. λ_{fixed} is obtained with the usual relationship $\delta_{fixed} = \frac{\delta_s - \Delta}{\Delta + 1}$ and Equation 1, where Δ is obtained before feeding by online isotopic measurements (Evans et al., 1986). Substituting Equation 2 in Equation 3 gives:

$$r_{day} = \frac{d}{SV_M} \times \frac{c_o\lambda_o - c_e\lambda_e + (c_e - c_o)\lambda_{fixed}}{\lambda_s - \lambda_{fixed}}. \quad (4)$$

It should be noted that possible $^{12}C/^{13}C$ fractionations that can occur during the absorption of labeled compounds are neglected here. The effect of such a fractionation is clearly negligible, that is, in the per mil order of magnitude, compared to the labeling level, which is in the percent order of magnitude or more.

Case of Feeding with a Compound That Has a Nonhomogeneous Positional Isotope Composition

The underlying assumption of the previous paragraph is that the substrate is isotopically homogeneous. However, the isotope composition of the feeding substrates used in these experiments is nonhomogeneous so that its different carbon atom positions do not have the same Δ values. In such a case, the metabolic reactions that are responsible for the decarboxylation of the carbon atoms and their rates should be taken into account. For example, this occurs when Glc or pyruvate is added: the C-1 atom of pyruvate is decarboxylated by pyruvate dehydrogenase, while the C-2 and C-3 positions are decarboxylated by the Krebs cycle (Fig. 1). Similarly, the C-3 and C-4 atom positions of Glc are decarboxylated by the pyruvate dehydrogenase reaction, the other being decarboxylated by the Krebs cycle. Advantage can then be taken from this with positionally ^{13}C-enriched substrates; ^{13}C-1-pyruvate would specifically enrich the CO_2 produced by pyruvate dehydrogenase, while ^{13}C-2-pyruvate would specifically enrich the CO_2 that comes from the Krebs cycle. The same applies to positional ^{13}C-enrichment in Glc.

The additional decarboxylations through the pyruvate dehydrogenase (PDH) reaction and the Krebs cycle are denoted as r_{PDH} and r_K, respectively (Fig. 1). With the relationship $r_{day} = r_{PDH} + r_K$, Equation 2 still works. For ^{13}C-1-pyruvate, Equation 3 becomes:

$$c_e\lambda_e + \frac{SV_M}{d}(\lambda_1 r_{PDH} + \lambda_c r_K) = c_{fixed}\lambda_{fixed} + c_o\lambda_o, \quad (5)$$

where λ_1 is the ^{13}C percentage in the C-1 position of the labeled pyruvate. λ_c is the ^{13}C percentage of the other (unlabeled) positions. For ^{13}C-2-pyruvate, we have:

$$c_e\lambda_e + \frac{SV_M}{d}\left(\lambda_c r_{PDH} + \frac{\lambda_c + \lambda_2}{2} r_K\right) = c_{fixed}\lambda_{fixed} + c_o\lambda_o, \quad (6)$$

where λ_2 is the ^{13}C percentage in the C-2 (labeled) position of pyruvate. λ_c (unlabeled positions) is the same as λ_c of Equation 5 (^{13}C-1 enrichment). It can be seen that the two conditions of ^{13}C-enrichment give two Equations (5 and 6) so that r_{PDH} and r_K can be deduced by a substitution procedure. A similar procedure is used for the positional ^{13}C-enrichment of Glc.

Rate of ^{13}C-Enriched Substrate Decarboxylation in Darkness

The CO_2 that is produced in darkness after a light period with ^{13}C-enriched substrate feeding comes from respiratory oxidation of new photosynthates (^{13}C percentage in the fixed carbon λ_{fixed}), photosynthates from the previous light period in the greenhouse (^{13}C percentage $\lambda_{previous}$), and additional C coming from the ^{13}C-enriched substrate fed to the leaf (^{13}C percentage λ_s). It has been previously shown that the contribution of new photosynthates to dark respiration after 3-h light in French bean is 40% (Nogués et al., 2004). So the ^{13}C percentage in photosynthates feeding respiration is given by $\lambda_p = 0.4\ \lambda_{fixed} + 0.6\ \lambda_{previous}$. It should be noted that possible variations in the coefficients due to some physiological reasons do only introduce a minor error in the estimate of the ^{13}C-enriched substrate decarboxylation r_{night} because of the strong ^{13}C-enrichment in the substrate.

The total CO_2 production in the dark is denoted as R_n. The ^{13}C percentage in dark respired CO_2 (denoted as λ_{global}) is calculated with the $\delta^{13}C$ value and Equation 1. The ^{13}C mass balance gives the following relationship:

$$R_n\lambda_{global} = r_{night}\lambda_s + (R_n - r_{night})\lambda_p. \quad (7)$$

Rearranging, it gives:

$$r_{night} = R_n \times \frac{\lambda_{global} - \lambda_p}{\lambda_s - \lambda_p}. \quad (8)$$

When substrates do not have a homogeneous isotopic distribution (positional enrichment), Equation 7 is completed to:

$$R_n\lambda_{global} = (r_{PDH}\lambda_1 + r_K\lambda_c) + (R_n - r_{PDH} - r_K)\lambda_p \quad (9)$$

for the ^{13}C-1-pyruvate feeding. And similarly, for the ^{13}C-2-enrichment, it gives:

$$R_n\lambda_{global} = \left(r_{PDH}\lambda_c + r_K \times \frac{\lambda_c + \lambda_2}{2}\right) + (R_n - r_{PDH} - r_K)\lambda_p. \quad (10)$$

Equations 9 and 10 allow one to extract r_{PDH} and r_K with a substitution procedure. The method is similar for ^{13}C-Glc.

RESULTS

On-Line Carbon Isotope Discrimination of Leaves Fed with ^{13}C-Enriched Substrates

The carbon isotope discrimination during photosynthesis of detached French bean leaves before and after addition of ^{13}C-enriched carbohydrates is shown in Figure 2. Before adding substrates, the carbon isotope discrimination was around 20‰ in all cases. This is in accordance with the p_i/p_a value around 0.7 (data not shown). At t = 60 min, ^{13}C-enriched substrates were added. The overall signature of Glc was 5,500‰ and that of pyruvate was 2,500‰. When $^{13}C_1$-pyruvate was supplied, the carbon isotope discrimination then increased to Δ_{obs} = 180‰ as a consequence of $^{13}C_1$-enriched pyruvate decarboxylation. The carbon isotope discrimination then slightly decreased and reached approximately 140‰. The on-line carbon isotope discrimination value increased much less with $^{13}C_2$-enriched pyruvate, with a maximum value of Δ_{obs} around 50‰, indicating that decarboxylations following Pyr dehydrogenation (i.e. Krebs cycle) had very small rates. Glc was hardly decarboxylated in the light, thus with a very small increase in Δ_{obs} (up to 35‰–40‰ only).

Rates of Decarboxylation in the Light

The rates of decarboxylation of the ^{13}C-enriched substrates (see Fig. 1 that summarizes the parameters considered) in the light were calculated using the carbon isotope discrimination Δ_{obs} and are given in Table I. These calculations used a two variable model (see ''Theory'' section) that is, r_{PDH}, the rate of decarboxylation through the PDH, and r_K, the decarboxylation through the Krebs cycle. These rates may be compared to the overall R_d. As expected from conclusions of Figure 2, the decarboxylation rate of pyruvate through the PDH reaction was around 0.05 to 0.06 μmol m^{-2} s^{-1}, the other decarboxylation rates being very low, under 0.01 μmol m^{-2} s^{-1}. Although the effect of decarboxylation of $^{13}C_1$-enriched Pyr was striking in Figure 2, the decarboxylation rate was low compared to day respiration (R_d, around 0.6 μmol m^{-2} s^{-1}) simply because the labeling was strong in Pyr. It is noteworthy that the decarboxylation rates were negligible compared to the overall R_d, so that we argue that the respiratory pathway was not artefactually enhanced in our feeding experiment. Accordingly, the overall R_d of fed leaves was similar to that of the control. Nevertheless, the decarboxylation CO_2 flux could have been somewhat underestimated because of refixation (see below).

Rates of Decarboxylation in Darkness

After 180 min (see Fig. 2), light was switched off and the carbon isotope composition of the CO_2 evolved in the first 30 min of darkness was measured. The δ^{13}C value of respired CO_2 with $^{13}C_1$- and $^{13}C_3$-Glc feeding was approximately 740‰ and 640‰, respectively, and the δ^{13}C value of respired CO_2 with $^{13}C_1$- and $^{13}C_2$-pyruvate feeding was approximately 520‰ and 290‰, respectively (Fig. 2, right). The decarboxylation rates calculated using these values are shown in Table I. r_{PDH} was around 0.070 μmol m^{-2} s^{-1} with either Glc or pyruvate and r_K with Glc was around the double (0.2 μmol m^{-2} s^{-1}) of that with pyruvate. Clearly, these decarboxylations witness that leaves oxidized molecules via glycolysis in the dark. A similar result was already obtained by Stitt and ap Rees (1978) with $^{14}CO_2$. When compared to the day decarboxylation values, the inhibition by light was around 95% for both r_{PDH} and r_K with Glc and around 30% and 95% for r_{PDH} and r_K with pyruvate, respectively. In other words, light inhibited only partially the pyruvate dehydrogenase reaction and almost stopped the Krebs cycle. In the light, the respiratory breakdown of Glc into CO_2 did not occur through the Krebs cycle or the pyruvate dehydrogenase reaction, simply demonstrating that the Glc molecules could not reach these metabolic steps in illuminated leaves.

Metabolic Pathways That Consumed ^{13}C-Enriched Glc

The weakness of Glc or pyruvate decarboxylation in the light raises the question of the fate of these molecules in the leaf. That is why starch purification on sample of the experiment of Figure 2 was made and NMR analysis of illuminated leaves fed with positional, fully ^{13}C-labeled substrates (99% ^{13}C in a given position) was done. The results are shown in Table II and Figure 3, respectively.

^{13}C-enriched Glc supplied to leaves was directed to Suc synthesis; the ^{13}C content of Suc as a whole

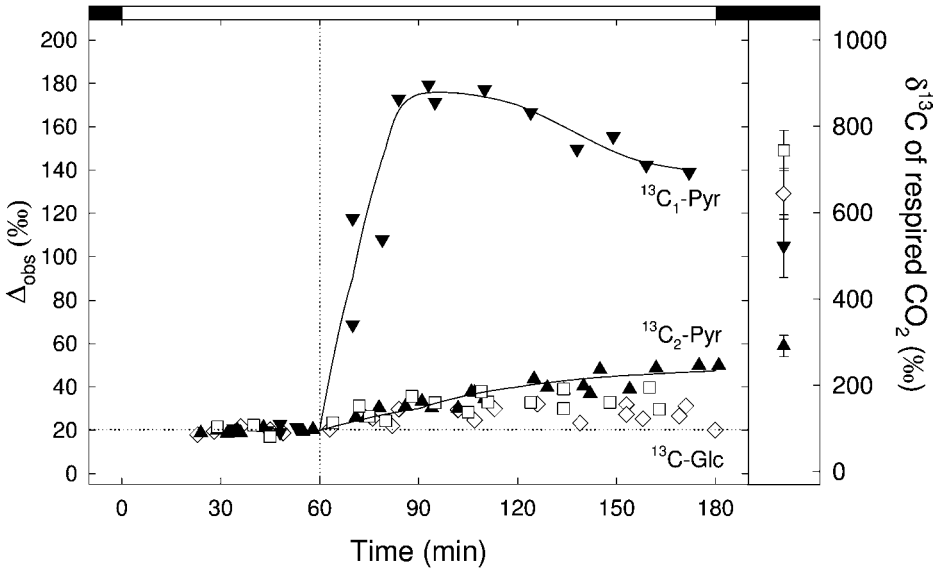

Figure 2. Left, Development of the on-line carbon isotope discrimination (Δ_{obs}) of detached bean leaves before and after feeding with Glc that is ^{13}C-enriched in C-1 (◇) or C-3 (□), or pyruvate that is ^{13}C-enriched in C-1 (▼) or C-2 (▲). In both positional enrichment cases, the overall δ^{13}C values of Glc and pyruvate are 5,500‰ and 2,500‰, respectively. The detached leaves are first put in distilled water and the light is turned on. The vertical dotted line indicates the moment at which the substrate (Glc or pyruvate) is supplied (t = 60 min). The horizontal dotted line represents the mean photosynthetic fractionation before substrate feeding (Δ_{obs} approximately equal to 20‰). The gas exchange conditions were 350 μL L^{-1} CO_2 in 21% O_2, 22°C, and 450 μmol m^{-2} s^{-1} light. Note that the Δ_{obs} measurements only begin after photosynthesis stabilizes that is, after approximately 30 min in the light. At t = 180 min, the light is switched off for dark-respired CO_2 measurements. The trends of the on-line isotope discrimination with Pyr feeding are indicated with a solid line. Right, δ^{13}C value (in per mil) of the CO_2 respired in the dark just after having switched off the light. Same symbols as for the left section.

approached 60% in both (C-1 and C-3) enrichment conditions (Fig. 3, A and B). When $^{13}C_3$-Glc was used, the scrambling of carbon atoms appeared, with a small labeling of all the carbon atom positions in the Glc moiety of Suc (^{13}C content around 4%). This presumably came from the pentose phosphates cycle. Accordingly, this effect was not seen with $^{13}C_1$-Glc because the first steps of the cycle are the dehydrogenation and decarboxylation of the C-1 carbon atom of Glc.

Surprisingly, starch was also labeled by Glc (Table II); with $^{13}C_1$-Glc and $^{13}C_3$-Glc, the δ^{13}C value of starch was strongly higher (−1.9‰ and −9‰, respectively) than in unfed leaves (−31.4‰). In other words, Glc could feed starch synthesis. Its contribution should have nevertheless been low as the starch amount was not significantly different between fed and unfed leaves (Table II). The contribution of feeding Glc (in percent of total starch amount), denoted here as σ, can be calculated with the following mass-balance relationship:

$$\delta^{13}C_{\text{with feeding}} = \sigma \times \delta^{13}C_{\text{feeding Glc}} + (1-\sigma) \times \delta^{13}C_{\text{without feeding}}$$

Table I. *Respiration rate and calculated decarboxylations (in μmol m^{-2} s^{-1}) of Glc and pyruvate supplied to detached French bean leaves through the PDH and the Krebs cycle in the light and in the dark (see "Theory" section for calculation details)*

Day respiration rates were measured as the slope of $\Gamma/r_i R_n$ relationships (see "Gas Exchange Measurements" section) and are thus total day respiratory fluxes in the light. Decarboxylation data are mean and SE of three independent measurements. The mean inhibition of decarboxylation by light was calculated as the ratio mean light decarboxylation/mean dark decarboxylation.

		Control	Pyruvate	Glc
Light				
R_d	Day respiration rate	0.632 ± 0.125	0.625 ± 0.150	0.539 ± 0.143
r_{PDH}	Decarboxylation from the PDH reaction		0.058 ± 0.009	0.004 ± 0.001
r_K	Decarboxylation from the Krebs cycle		0.005 ± 0.004	0.009 ± 0.004
Darkness				
R_n	Dark respiration rate	1.178 ± 0.039	1.266 ± 0.071	1.331 ± 0.115
r_{PDH}	Decarboxylation from the PDH reaction		0.079 ± 0.023	0.070 ± 0.015
r_K	Decarboxylation from the Krebs cycle		0.116 ± 0.011	0.236 ± 0.050
i_{PDH}	Mean inhibition of the PDH reaction by light		27%	94%
i_K	Mean inhibition of the Krebs cycle by light		95%	96%

Table II. *Amount and carbon isotope composition ($\delta^{13}C$) of starch from detached leaves supplied with ^{13}C-enriched Glc ($\delta^{13}C$ 5,500‰) or ^{13}C-enriched Pyr ($\delta^{13}C$ 2,500‰), 15 mmol L^{-1} for 2 h in the light with 350 $\mu L\ L^{-1}$ CO_2 at −51‰ (see Fig. 1) after 1 h in the light without feeding*

Data are mean and SD of three independent measurements.

Conditions	Amount	$\delta^{13}C$
	$\mu g\ mg^{-1}$ fresh weight	*‰*
Control (greenhouse)	13.1 ± 5.3	−28.5 ± 0.5
No feeding	15.1 ± 5.0	−31.4 ± 1.3
$^{13}C_1$-Glc	15.0 ± 3.3	−1.9 ± 9.0
$^{13}C_3$-Glc	13.6 ± 2.9	−9.0 ± 9.5
$^{13}C_1$-Pyr	11.7 ± 5.4	−16.5 ± 3.4
$^{13}C_2$-Pyr	12.2 ± 3.2	−29.9 ± 1.6

that gives σ = 0.5% ± 0.2% and 0.4% ± 0.2% for $^{13}C_1$-Glc and $^{13}C_3$-Glc, respectively. This contribution was very low, but was clearly seen in starch (Table II) simply because the $\delta^{13}C$ value of the labeling Glc was very high. This result indicates that Glc molecules from the cytoplasm (or, more generally, C from the fed Glc) could reach the chloroplastic compartment and enter the starch synthetic pathway.

The enriched carbon atoms of Glc were almost not redistributed to other positions by metabolic pathways; when supplied with $^{13}C_1$-Glc, the C-6 carbon atoms in leaf Glc, Fru, or Suc were not labeled (Fig. 3A). Similarly, when supplied with $^{13}C_3$-Glc, the C-4 carbon atoms in leaf Glc and Fru were not labeled and the corresponding positions in Suc were only weakly labeled (4% of the ^{13}C content; Fig. 3B). In other words, the scrambling of carbon atoms through the triose phosphates and hexose phosphates interconversion (with triose phosphate isomerase and aldolase) was very low. This result is consistent with the gas exchange measurements of Figure 2 in which glycolysis appeared to be stopped so that Glc was not (or very weakly) decarboxylated.

Metabolic Pathways That Consumed ^{13}C-Enriched Pyruvate

When leaves were fed with $^{13}C_2$-Pyr (99% of ^{13}C in C-2), Ala was (weakly) labeled in C-2, indicating that Pyr had been aminated and citrate was labeled, strongly suggesting that some of the Pyr molecules entered the Krebs cycle (Fig. 3D). Surprisingly, when fed with $^{13}C_1$-Pyr, leaves had only a few labeled carbon atoms (Fig. 3C). Ala appeared as only weakly labeled in C-1, but this originated mainly from the low detectability of carboxyl (−COOH) carbon atoms with NMR. Moreover, the CO_2 produced in the light accounted for an important ^{13}C loss; using a decarboxylation rate of 0.05 $\mu mol\ m^{-2}\ s^{-1}$ (Table I), there was a ^{13}C loss of 350 $\mu mol\ ^{13}C\ m^{-2}$ during the light treatment (2 h),while approximately 1,950 μmol Pyr m^{-2} entered the leaf (=Pyr concentration × transpiration rate = 15 $mol\ L^{-1}$ × 18 $\mu L\ m^{-2}\ s^{-1}$ × 2 h light), that is, approximately 18% of the ^{13}C supplied in C-1. A similar calculation gives, with $^{13}C_2$-Pyr, a ^{13}C loss of only approximately 1%.

Refixation of Decarboxylated CO_2

The rate of the decarboxylation of ^{13}C-enriched substrates in the light may have been underestimated by possible refixation of CO_2. If so, the absolute rate of refixation is expected to be important when $^{13}C_1$-Pyr was supplied to leaves as decarboxylation was high in that case (Table I). Nevertheless, Glc and Fru were not labeled and Suc was only weakly labeled in the C-3 position of the Fru moiety (Fig. 3C). Although it is difficult to quantify such small quantities, this labeling in Suc accounted, as a maximum, for 0.5 $\mu mol\ g^{-1}$ of ^{13}C, that is, a ^{13}C (re)fixation rate of 0.009 $\mu mol\ m^{-2}\ s^{-1}$ during 2 h of the treatment in the light. When $^{13}C_1$-Pyr was supplied to leaves, starch was also labeled (the isotope signature is around −20‰, compared to −31‰ expected; Table II) and this was also a consequence of refixation. The amount of ^{13}C-enriched carbon in starch was calculated to be 0.2% (similar calculation as for section "Metabolic Pathways That Consumed ^{13}C-Enriched Glc"). The starch amount and the percentage of carbon in starch were 0.015 mg mg^{-1} fresh weight and approximately 40%, respectively. This gives a refixation rate (of labeled carbon) of 0.008 $\mu mol\ m^{-2}\ s^{-1}$. The overall refixation rate would thus have been, as a maximum, 0.017 $\mu mol\ m^{-2}\ s^{-1}$. This means that the total decarboxylation rate of $^{13}C_1$-Pyr would have been 0.058 (see Table I) + 0.017 = 0.075 $\mu mol\ m^{-2}\ s^{-1}$ and so, the inhibition of the PDH reaction by light would have been approximately 5% only. The effect of refixation in the other case ($^{13}C_2$-Pyr) was clearly negligible: starch was not labeled (Table II) and no labeling could be seen in Suc, Glc, or Fru (Fig. 3D). As the rates of decarboxylation were similar with C-1 and C-3 ^{13}C-enriched Glc, refixation was likely to be negligible in both cases.

DISCUSSION

The respiratory metabolism in illuminated leaves is inhibited compared to darkness, as repeatedly shown by gas-exchange measurements (for review, see Atkin et al., 2000). However, the metabolic basis of such an inhibition is not well known. We addressed this question by feeding experiments using ^{13}C-enriched substrates and followed the ^{13}C atoms with isotope ratio mass spectrometry and NMR to determine which metabolic pathways are inhibited in the light.

The Pyruvate Dehydrogenase Activity in the Light

The two main steps responsible for the respiratory CO_2 production are the dehydrogenation of Pyr (PDH reaction) and the Krebs cycle (Fig. 1). The carbon atoms that are decarboxylated are not the same in

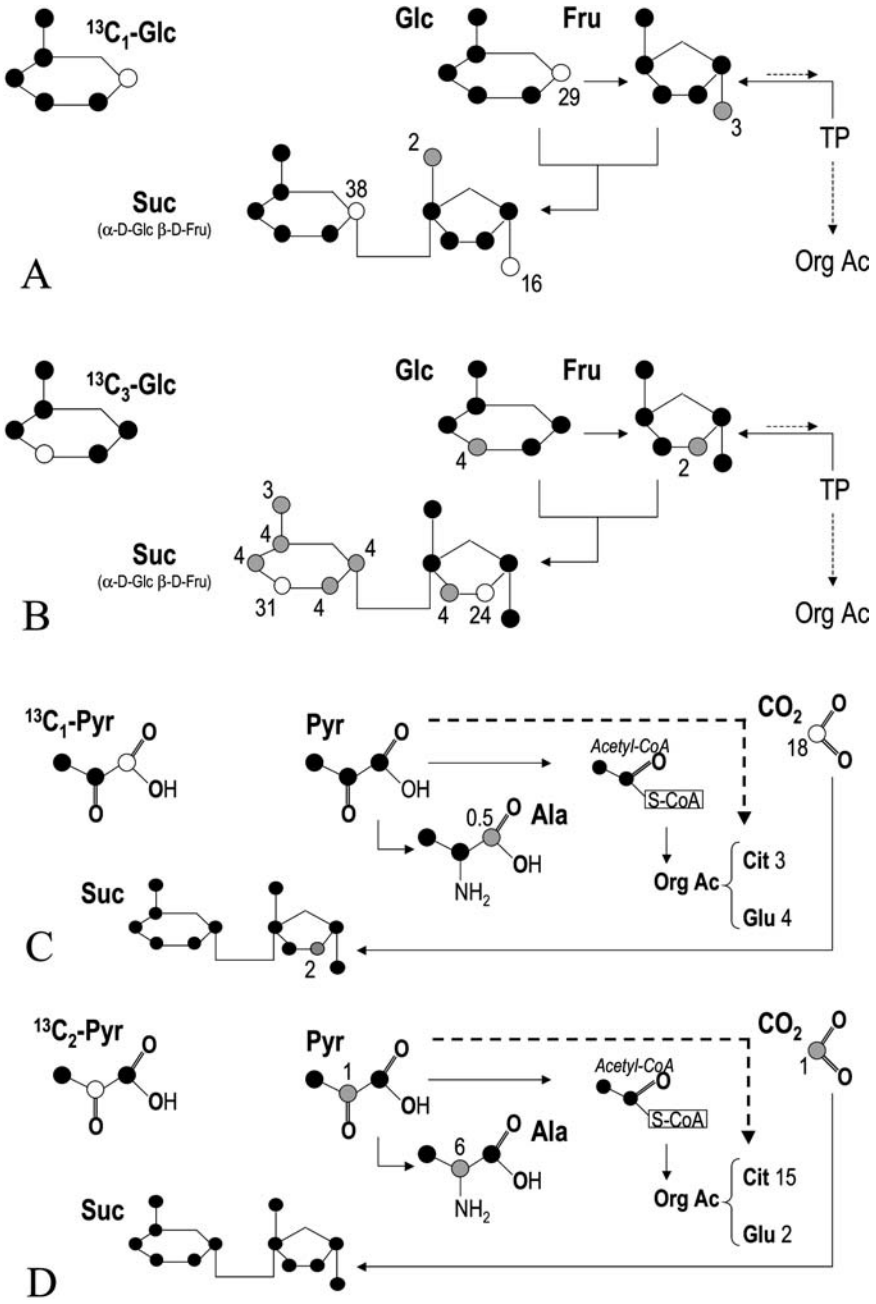

Figure 3. Distribution of ^{13}C from NMR analysis of leaves fed with $^{13}C_1$- or $^{13}C_3$-Glc (A and B), $^{13}C_1$- or $^{13}C_2$-pyruvate (C and D) for 2 h in the light at ambient CO_2. In each case, the compound supplied to leaves is indicated in the top left of each section. Simplified metabolic pathways are represented in order to emphasize the relationships between metabolites. Cit, Citrate; TP, triose phosphates; Org Ac, organic acids. The dashed line stands for the production of organic acids from pyruvate via the phospho*enol*pyruvate carboxylation. The bottom continuous line in C and D stands for the refixation of decarboxylated CO_2. Carbon atoms are colored in accordance with their labeling level: black, no labeling, gray, low labeling, white, strong labeling. For each labeled carbon, numbers are in percent of ^{13}C that is in the sample. Carbon atoms that are considered to be labeled are those which proportion of ^{13}C ($^{13}C/^{12}C + ^{13}C$) is equal or more than 2% (the natural abundance of ^{13}C is approximately 1.1%).

both: the PDH reaction decarboxylates the C-1 of Pyr, while the Krebs cycle decarboxylates the two others. Feeding illuminated leaves with ^{13}C-enriched Pyr in C-1 significantly enriched the respiratory CO_2 produced in the light, as revealed by the strong modification of the photosynthetic carbon isotope discrimination measured on-line, from the steady value of 20‰ to approximately 160‰ (Fig. 2). Clearly, the PDH reaction consumes the Pyr molecules, and so is not totally inhibited in the light. When compared to the dark decarboxylation rate, the calculated inhibition is 27% (Table I). Refixation of decarboxylated CO_2 can nevertheless occur and lead to an overestimation of the inhibition level. Indeed, there was a small ^{13}C enrichment in Suc (Fig. 3) as well as in starch (Table II) and a calculation (see ''Results'') gives a refixation rate of 0.017 μmol m^{-2} s^{-1}; that is, a total decarboxylation rate of Pyr of 0.075 μmol m^{-2} s^{-1}. The refixation rate obtained here stands for 22% of the (total) decarboxylation of ^{13}C-enriched Pyr, a value that is in accordance with calculations that use ^{14}C data by Gerbaud and André (1987) on sunflower leaves (between 15% and 22%), but a little low compared to Pinelli and Loreto (2003) who found a refixation rate of 40% in mint leaves maintained at 350 μL L^{-1}. In this study, we looked at only ^{13}C enrichment in the two main

compounds of CO_2 fixation; that is, Suc and starch. Nevertheless, we recognize that some ^{13}C atoms in other compounds were missed, leading to a small underestimation of the refixation rate. Taking our value as correct, this would give a light inhibition value of the PDH reaction of only 5%.

However, it is not possible in our experiment to distinguish the chloroplastic PDH activity from the mitochondrial one so that our values do represent the total cellular PDH activity. It has been shown that the mitochondrial PDH is partly inhibited in illuminated leaves by phosphorylation (Budde and Randall, 1990). In addition, photorespiratory produced NH_3 is also assumed to inhibit this enzyme (Krömer, 1995). By contrast, the chloroplastic PDH is not regulated by phosphorylation and is assumed to be active in the light (Plaxton, 1996). The decarboxylation rate measured in this study (Table I) is likely to be the sum of both PDH activity in which the chloroplastic enzyme plays the major role.

Furthermore, the PDH reaction may be artefactually activated by the amount of Pyr (thermodynamic mass action law) introduced in the leaf. We nevertheless think that this effect is minor; the pyruvate concentration in the feeding solution is only 15 mmol L^{-1} and although it is difficult to detect carboxyl carbon atoms by NMR, it was not possible to see the corresponding C-1 atom of Pyr on the NMR spectrum, indicating that the Pyr concentration in the leaf is certainly less than 2 mmol L^{-1} in the cell.

Glycolysis and the Krebs Cycle Are Inhibited in the Light

In contrast with $^{13}C_1$-Pyr, when leaves were fed with $^{13}C_2$-Pyr, the photosynthetic carbon isotope discrimination was hardly modified (Fig. 2), indicating that there was nearly no decarboxylation of the C-2 carbon atom of Pyr. In other words, the Krebs cycle activity was very low and so there is a very small $^{13}CO_2$ production. The calculated decarboxylation rate is 0.005 μmol m^{-2} s^{-1} only in the light; that is, the Krebs cycle is inhibited by 95% compared to the dark decarboxylation rate (Table I). This is consistent with the NMR results: (1) the intermediary products of the Krebs cycle such as succinate and fumarate are not detected, Glu (that is the amino acid that corresponds to α-ketoglutarate) was hardly labeled, (2) the two carboxyl atoms of citrate were ^{13}C-labeled so that citrate stood for 15% of the sample ^{13}C content, i.e. each carboxyl carbon atom represented 7.5% only of the ^{13}C content. These data are in accordance with previous observations of Hanning and Heldt (1993) that mitochondria extracted from illuminated leaves had a low metabolic flux throughout the Krebs cycle. In addition, the mitochondrial matrix in the light is supposed to be reduced because of the photorespiratory Gly decarboxylation that lead to a high NAD(P)H/$NAD(P)^+$ ratio. Some enzymes of the Krebs cycle are inhibited by the high NADH/NAD^+ ratios (for review, see Siedow and Day, 2000), and it has been recently found that isocitrate dehydrogenase from pea is inhibited by high NADPH/$NADP^+$ ratios, a feature that occurs in illuminated mitochondria (Igamberdiev and Gardeström, 2003).

The fact that the Krebs cycle is slowed down in the light raises the question of the fate of acetyl-CoA molecules produced by the PDH reaction. In our study, acetyl-CoA probably accumulated a little: the decrease in the photosynthetic on-line discrimination after 115 min (Fig. 2) suggests that the PDH reaction was less active. This was likely because of the retroinhibition of Pyr dehydrogenase by its product acetyl-CoA (Harding et al., 1970; Rapp et al., 1987), as the Krebs cycle activity that consumes acetyl-CoA was severely diminished in the light. A significant part of the acetyl-CoA molecules was directed to fatty acid production in the chloroplast (Ohlrogge and Jaworski, 1997). Accordingly, the mutant line of Arabidopsis (*Arabidopsis thaliana*) that produces the antisense RNA of the PDH kinase (thus enhancing the mitochondrial PDH reaction), accumulated ^{14}C-labeled fatty acids when ^{14}C-Pyr was fed to (photosynthetic) stems (Marillia et al., 2003), strongly suggesting that fatty acid synthesis can act as outfall for acetyl-CoA molecules.

^{13}C-enriched Glc was only weakly decarboxylated in the light whatever the position of the ^{13}C-enrichment was (Fig. 2). Thus, Glc molecules could hardly reach the PDH step, very likely because they could not enter glycolysis. Instead, the Glc molecules were directed to Suc synthesis (Fig. 3). Further, when fed with $^{13}C_1$-Glc, the Fru moiety in Suc was only very weakly labeled in C-6, and similarly, when fed with $^{13}C_3$-Glc, the Fru moiety in Suc was hardly labeled in C-4 (Fig. 3). Clearly, this shows that the C-1/C-6 and C-3/C-4 interconversion through the triose phosphates isomerase reaction was minor. In other words, only a small fraction (less than approximately 5% of the ^{13}C-Glc fed to the leaf) of the Glc molecules reached this step, strongly suggesting a high metabolic resistance to the glycolytic breakdown of hexoses. Noteworthy, these results are in accordance with the regulation of the enzymes responsible for the phosphorylation/dephosphorylation of Fru-6-P to Fru-2,6-bisphosphate: (1) in the light, the high triose phosphates/inorganic phosphate decreases the Fru-2,6-bisphosphate concentration, promoting the dephosphorylation of Fru-1,6-bisphosphate in the cytosol (Stitt, 1990), and (2) in the chloroplast, phosphofructokinase is thought to be inhibited in the light (Plaxton, 1996). The rationale of such enzymatic regulations is that hexose molecules are prevented from entering the glycolytic breakdown, and so respiration does not consume Suc as soon as it is synthesized in the cytoplasm.

Cytoplasmic Glc Contributes to Starch Synthesis in the Light

When fed with ^{13}C-enriched Glc (at 5,500 per mil), leaves produced ^{13}C-labeled starch (Table II), with

a $\delta^{13}C$ value around 30‰ higher than without Glc feeding, indicating that Glc contributed to starch synthesis. The contribution was nevertheless low, around only 0.5% of the starch amount (that is, approximately 0.075 μg mg^{-1} fresh weight) was labeled (see the "Result" section for calculations). The flux of starch synthesis from ^{13}C-enriched Glc during the 2 h of feeding treatment in the light was then 0.075 μg mg^{-1}/2 h $\sim$ 0.038 μg mg^{-1} h^{-1}; that is, 0.045 μmol C m^{-2} s^{-1}, while the total starch synthetic flux was around 2 μg mg^{-1}/3 h $\sim$ 0.7 μg mg^{-1} h^{-1} (Table II); that is, 0.84 μmol C m^{-2} s^{-1}. So, Glc fed 0.045/0.84 $\sim$ 5% of starch synthesis in the leaf during the feeding experiment. The rate of starch synthesis from ^{13}C-enriched Glc is consistent with that found in potato by Quick et al. (1995): chloroplasts extracted from intact leaves and illuminated with ^{14}C-Glc (in a medium containing HCO_3^-) synthesized starch from ^{14}C-Glc with a rate of approximately 0.04 μmol C m^{-2} s^{-1} (recalculated assuming a realistic chlorophyll amount of 0.4 g Chl m^{-2}).

It has also been found that other carbon sources than photosynthetic CO_2 can feed starch synthesis in intact illuminated leaves: Nogués et al. (2004) showed that it was not possible to completely label starch with CO_2 and while the starch amount increased, the $\delta^{13}C$ value of starch reached a plateau.

These observations might be paralleled with the presence of a diffusion-driven Glc phosphate translocator on the inner chloroplast membrane (Schäfer et al., 1977; Quick et al., 1995), which can feed starch synthesis with cytoplasmic hexose molecules, although the associated flux appears to be quantitatively minor in this study.

Concluding Remarks

To our knowledge, this paper describes the first in vivo study on the metabolic basis of inhibition by light of leaf respiration. Clearly, the main inhibited steps are the entrance of hexose molecules into the glycolytic pathway and the Krebs cycle. We nevertheless recognize that our experiments were made in typical conditions (21% O_2, 350 μL L^{-1} CO_2, 22°C) and the results may be influenced by environmental parameters. Indeed, it has been shown that there lies no inhibition of respiration by light in some physiological conditions (Sharp et al., 1984, and refs. therein). Two parameters are of particular interest: temperature, which is known to enhance respiratory enzymatic activities, and the photorespiratory rate (oxygen partial pressure), which has an effect on the redox status of the mitochondria. Further experimental data are now needed to investigate the effect of such environmental conditions on day respiratory metabolism. Moreover, the fact that the Krebs cycle appears to be slowed down in the light raises the question of energy in leaf cells and more precisely, of NADH feeding of the respiratory chain. Although one may suggest that photorespiration or the cytoplasmic-mitochondrial malate shuttle have such a role, further metabolic studies are needed to determine their respective contribution in vivo.

MATERIALS AND METHODS

Plant Material

French bean (*Phaseolus vulgaris*) L. cv Contender plants were grown from seed in 1-L pots of potting mix in a greenhouse, as described by Tcherkez et al. (2003). Minimum photosynthetic photon flux density during a 16-h photoperiod was maintained at approximately 500 μmol m^{-2} s^{-1} by supplemental lighting. Temperature and vapor pressure deficit were maintained at approximately 25.5°C/18.5°C and 1.4/1.2 kPa day/night, respectively. Carbon isotope composition ($\delta^{13}C$) of CO_2 in the greenhouse air was $-9.5‰ \pm 0.3‰$. The first trifoliar fully expanded leaves were used for all measurements.

Gas Exchange Measurements

Closed System (Dark Respiration)

The respiration chamber was placed in a closed system, which was directly coupled to an elemental analyzer (EA) NA-1500 (Carlo-Erba, Milan) through a 15-mL loop, as described by Tcherkez et al. (2003). Briefly, molar fractions of respiratory CO_2 were measured with an infrared gas analyzer (IRGA; Finor, Maihak, Germany) placed in the closed system that was first flushed with CO_2-free air. The loop was shunted when CO_2 reached around 300 μL L^{-1} and the gas inside the loop was introduced into the EA with helium for gas chromatography. The connection valve between the elemental analyzer and the isotope ratio mass spectrometer (VG Optima, Micromass, Villeurbanne, France) was opened when the CO_2 peak emerged from the EA.

Open System (Photosynthesis and On-Line Carbon Isotope Discrimination)

The assimilation chamber was connected in parallel to the sample air hose of the LI-6400 (LI-COR, Lincoln, NE). This aluminum chamber ([20 $\times$ 12 $\times$ 6] 10^{-6} m^3) had a clear Plexiglas lid that allowed us to accommodate the middle leaflet (typical leaf surface approximately 0.01 m^2). Two fans placed in the chamber gave a boundary layer conductance to water of approximately 6.7 mol m^{-2} s^{-1}. Leaf temperature was controlled at 20°C with circulating water from a cooling water bath to the jacket of the leaf chamber and was measured with a copper-constantan thermocouple plugged to the thermocouple sensor connector of the LI-6400 chamber/IRGA. Ingoing air was dried (at approximately 1 mmol H_2O mol^{-1}) and passed through the chamber at a rate of 1 L min^{-1}, monitored by the LI-6400. Molar fractions of CO_2 were measured with the IRGA of the LI-6400. Light was supplied by a 500-W halogen lamp (Massive N. V., Kontich, Belgium). The lamp was placed about 30 cm above the chamber and 5 cm of deionized water and 1 cm of glass in the container filtered the radiation. The photosynthetic photon flux density at leaf level inside the chamber was maintained at 450 μmol m^{-2} s^{-1} during the labeling period. Inlet CO_2 was obtained from a gas cylinder (Air Liquide, Grigny, France) with a $\delta^{13}C$ of $-51.2‰ \pm 0.2‰$. The outlet air of the chamber was regularly shunted and was sent to the loop to measure its isotope composition and thus the on-line carbon isotopic discrimination (Δ_{obs}). The gas inside the loop was introduced into the EA for gas chromatography as described above. Δ_{obs} during photosynthesis was measured following the method described by Evans et al. (1986; see "Theory").

Day Respiration Measurements

Day respiration was measured with a LICOR-6400 open system, according to the method described in Peisker and Apel (2001). Briefly, A/c_i curves are done at different light levels and the linear fit gives the CO_2 compensation point Γ and the internal leaf resistance r_i. Γ is plotted as a function of the product $r_i R_n$ and the slope of the linear regression is $\mu = R_d/R_n$ and so gives R_d. In bean, the effect of light on the Γ/r_i relationship was slight and did not modify significantly the estimate of μ.

Starch Extraction Procedure

The extraction procedures for starch were similar to that described by Tcherkez et al. (2003). Leaf powder was suspended with 1 mL of distilled water in an Eppendorf tube (Eppendorf Scientific, Hamburg, Germany). After centrifugation, the pellet was washed four times with 95% ethanol at room temperature and starch was extracted with HCl solubilization and precipitated with cold methanol. After lyophilization, starch is transferred to tin capsules (Courtage Analyze Service, Mont Saint-Aignan, France) for isotope analysis.

NMR

Perchloric acid (PCA) extracts were prepared from 5 g of frozen leaf material as described by Aubert et al. (1996) for phloem cells. Spectra were obtained on a spectrometer (AMX 400) equipped with a 10-mm multinuclear probe tuned at 161.9 and 100.6 MHz for ^{31}P- and ^{13}C-NMR, respectively. The deuterium resonance of $^{2}H_2O$ (100 μL added per mL of extract) was used as a lock signal.

Conditions for ^{13}C-NMR acquisition utilized 19-μs pulses (90°) at 6-s intervals and a sweep width of 20 kHz. Broad-band decoupling at 2.5 W during acquisition and 0.5 W during the delay was applied using the Waltz sequence; the signal was digitized using 32,000 data points zero-filled to 64,000 and processed with a 0.2-Hz line broadening. ^{13}C-NMR spectra are referenced to hexamethyldisiloxane at 2.7 ppm. Mn^{2+} ions were chelated by the addition of 1 mmol L^{-1} 1,2-cyclohexylenedinitrilotetraacetic acid. The assignments of resonance of ^{13}C peaks were carried out according to Gout et al. (1993). Identified compounds were quantified from the height of their resonance peaks using fully relaxed conditions for spectra acquisition (pulses at 20-s intervals). Peak intensities were normalized to a known amount of the reference compound (maleate) that is added to the sample (internal standard). A carbon atom is here considered to be labeled when its estimated positional $^{13}C/(^{13}C + ^{12}C)$ is more than 2% (the natural abundance is nearly 1.1%).

^{13}C-Enriched Molecules

The positional ^{13}C-labeled molecules (99% ^{13}C in the considered position) were purchased to Eurisotop (Saclay, France). Pyruvate was dissolved in distilled water and pH was corrected to 6.8 with NaOH. To obtain non-fully labeled solutions, the labeled compounds were mixed with industrial Glc ($\delta^{13}C = -9‰$) or pyruvate ($\delta^{13}C = -21‰$) from Sigma. The resulting overall composition of the Glc and pyruvate solutions was checked to be 5,500‰ and 2,500‰, respectively. In other words, the ^{13}C-enriched carbon atom position had a composition of 47,750‰ and 8,000‰, respectively (the other positions being at $-9‰$ and $-21‰$ for Glc and Pyr, respectively). The final concentration was 0.015 mol L^{-1} in all cases. The solutions were poured in an Eppendorf tube and fed to the leaves through the transpiration stream.

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