Tansley review

Leaf day respiration: low CO_2 flux but high significance for metabolism and carbon balance

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Summary

It has been 75 yr since leaf respiratory metabolism in the light (day respiration) was identified as a low-flux metabolic pathway that accompanies photosynthesis. In principle, it provides carbon backbones for nitrogen assimilation and evolves CO_2 and thus impacts on plant carbon and nitrogen balances. However, for a long time, uncertainties have remained as to whether techniques used to measure day respiratory efflux were valid and whether day respiration responded to environmental gaseous conditions. In the past few years, significant advances have been made using carbon isotopes, 'omics' analyses and surveys of respiration rates in mesocosms or ecosystems. There is substantial evidence that day respiration should be viewed as a highly dynamic metabolic pathway that interacts with photosynthesis and photorespiration and responds to atmospheric CO_2 mole fraction. The view of leaf day respiration as a constant and/or

negligible parameter of net carbon exchange is now outdated and it should now be regarded as a central actor of plant carbon-use efficiency.

I. Introduction

Studies of the influence of leaf gas-exchange properties on growth usually focus on photosynthetic physiology. However, CO_2 fixation by plants via photosynthesis is not a sufficient basis on which to predict growth as respiration, nitrogen (and sulphur) assimilation, amino acid synthesis and other processes (like losses through volatiles organic compounds and exudates) must also be considered. Neglecting these may result in an overestimation of crop yield by up to 30% relative to estimates based solely on a given amount of assimilated CO_2 (Penning De Vries, 1975). Improvement of crop yield potential requires identification of specific aspects of plant metabolism that can be manipulated to optimize efficiency. When nutrient, water and light supplies are sufficient to reach yield potential, production can be increased by improving efficiency (i.e. more carbon assimilated per unit nutrient taken up) (Lawlor, 2002). Metabolism may also be manipulated to increase the capacity for nutrient use. Simultaneous increase in both is desirable to maintain an appropriate C : N balance in plant organic matter. Therefore, an understanding of basic metabolic processes and how they relate to plant biomass production is essential. Leaf day respiration plays a central role in this relationship because nutrients are assimilated in illuminated leaves (at least in most crops), using carbon backbones produced via respiratory metabolism. Unfortunately, modelling and predicting carbon fluxes in day respiration remain difficult.

Leaf day respiration is usually defined in the gas-exchange literature as the rate of nonphotorespiratory CO_2 evolution in the light, expressed on a leaf area basis. This definition is coarse (at least, from a metabolic perspective) because it encompasses distinct metabolic pathways such as the 'Krebs cycle' (or tricarboxylic acid pathway, TCAP), the oxidative pentose phosphate pathway (PPP), and all other nonphotorespiratory decarboxylation reactions (e.g. malic enzyme activity, formate degradation, etc.). Alternatively, leaf respiration could be defined as the nonphotorespiratory oxygen (O_2) consumption. Again, this definition is problematic because it encompasses many processes that affect net oxygen consumption, such as reoxidation of NADH from photorespiratory glycine oxidation or from excess reductive power exported by the chloroplast. Because of its importance for plant carbon budget, we will adopt here the definition of 'respiration' based on central catabolism involving the glycolytic pathway and the TCAP, while recognizing that other CO_2-releasing reactions may play important roles in observed changes in what is typically and operationally defined as day respiration. In this review, we will summarize metabolic pathways involved in day respiration and discuss the impact of day respiratory efflux for leaf gas exchange, and thus its significance for plant carbon and nitrogen budget.

II. Pioneering metabolic studies of day respiration with ^{14}C

Respiratory metabolism during photosynthesis has been investigated for at least 70 yr. In the 1940s, Kok and co-workers provided evidence, using oxygen exchange measurements, that the O_2-consuming respiratory flux at low light was slower than the same flux in the dark (Kok effect) (Kok, 1948, 1949; see also Tcherkez *et al.*, 2017 for a specific discussion on the significance of the Kok effect). Despite a long period of subsequent research, the way in which respiratory carbon metabolism operates in the light remained controversial. In fact, in the first ^{14}C labelling studies on unicellular algae by Calvin and co-workers, no ^{14}C at all was found in TCAP intermediates, suggesting that respiratory metabolism was totally arrested in the light (Calvin & Massini, 1952). A few years later, similar experiments showed a small amount of ^{14}C in such intermediates or metabolites synthesized therefrom (Holm-Hansen *et al.*, 1959; Moses *et al.*, 1959). Furthermore, ^{14}C sugars were found not to be respired in illuminated leaves (Vittorio *et al.*, 1954). In illuminated wheat (*Triticum aestivum*) leaves, labelling with ^{14}C-glucose led to ^{14}C build-up in sucrose, and very small amounts of ^{14}C in downstream metabolites like glutamate and alanine (unlike the situation in the dark). This suggested that glucose could not enter glycolysis in the light. However, labelling with ^{14}C-glutamine led to redistribution of ^{14}C in glutamate, sugars, and organic acids, clearly showing that glutamine could be metabolized via the Krebs cycle (Bidwell *et al.*, 1955). At the time, such results appeared to be in contradiction with fatty acids (FA) being ^{14}C-labelled upon $^{14}CO_2$ feeding (in illuminated leaves and chloroplasts; Stumpf & James, 1962, 1963; Stumpf *et al.*, 1963), simply because FA production requires glycolytic degradation of glucose to pyruvate. Subsequent labelling with ^{14}C metabolites (including citrate or fumarate) suggested that the flux through the Krebs cycle decreased transiently upon the dark-to-light transition but operated at a similar rate in the light and in the dark (Graham & Walker, 1962; Chapman & Graham, 1974a,b). A typical difference between light and dark was that oxaloacetate appeared to be converted to malate in the light while it yielded aspartate in the dark. This was interpreted as being the result of excess reductive power slowing down the 'left branch' of the Krebs cycle (Graham & Walker, 1962; Chapman & Graham, 1974a). Experiments monitoring $^{14}CO_2$ evolution in CO_2-free air further suggested that respiratory CO_2 efflux was inhibited by *c.* 75% in the light (Mangat *et al.*, 1974).

In summary, the metabolic framework of day respiration had been mostly established by the mid-1970s. Key features were that glucose was prevented from entering glycolysis, and the flux pattern through the TCAP was modified (relative to dark conditions). Subsequent studies, including quantitative flux measurements, have further elucidated the processes, but these two points remain valid.

III. Metabolic flux pattern of day respiration

Considerable further advances have been made in the past 25 yr in describing metabolism associated with day respiration, and in reconciling important metabolic roles of TCAP activity (e.g. for nitrogen assimilation; see the following section) with its down-regulation (inhibition) in the light (summarized in Fig. 1).

1. Inhibition of respiration by light

Inhibition of respiratory CO_2 efflux in the light has been demonstrated by gas exchange using both Laisk and Kok methods (this has been extensively reviewed in Atkin *et al.*, 2000; see Box 1 for a summary of methods). Other methods that use $^{12}C/^{13}C$ isotopes (Pinelli & Loreto, 2003), efflux at Γ* (the compensation point in the absence of day respiration; Atkin *et al.*, 1998), CO_2

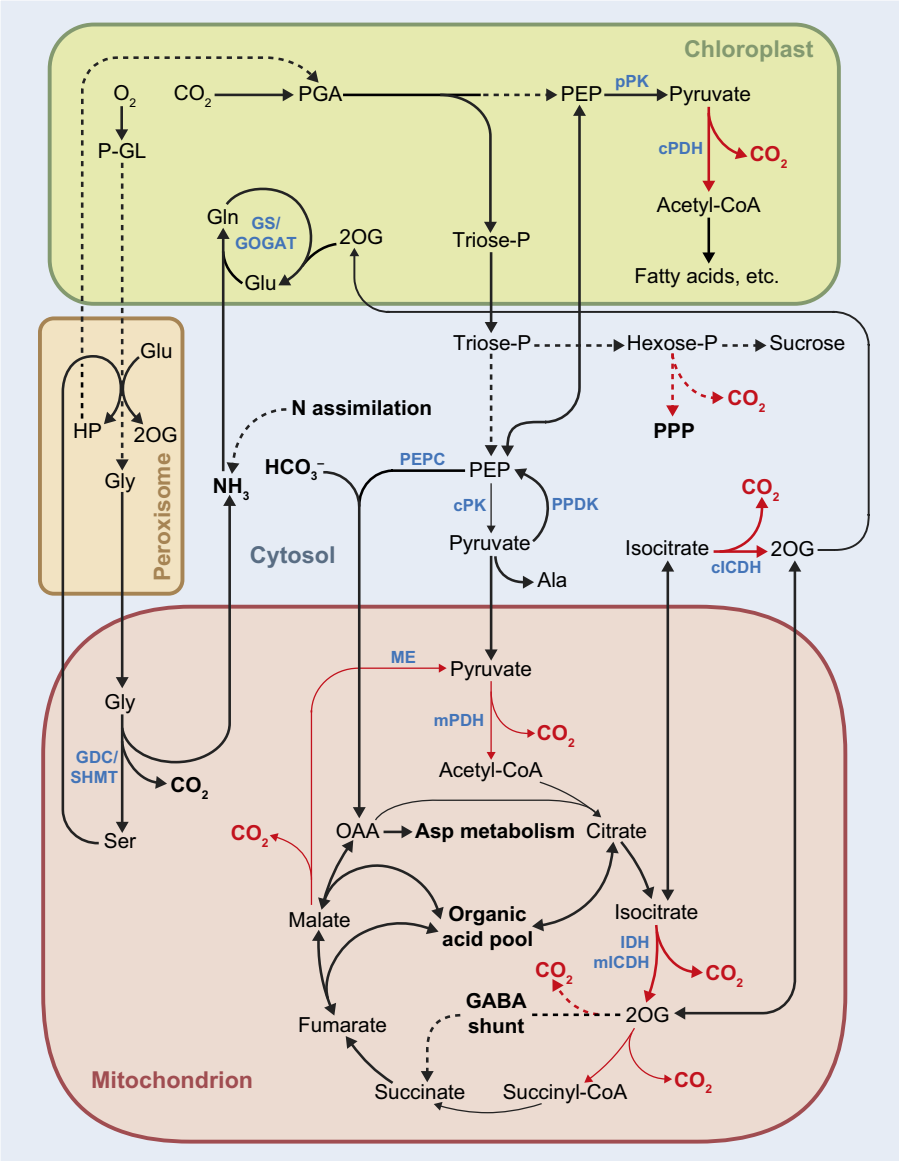

Fig. 1 Simplified carbon primary metabolism associated with day respiratory CO_2 release, showing major decarboxylation reactions (in red). Steps slowed down in the light (as compared with the dark) are shown with thin lines. Dashed lines stand for multiple steps. Enzymes indicated in blue are further discussed in the main text. Initial lowercase letters p, m and c stand for plastidic, mitochondrial, and cytosolic. For clarity, this scheme does not show redox metabolism (including OAA-malate shuttle between compartments) and simplifies the photorespiratory cycle. Note the absence of the PPP in the chloroplast as it is inhibited by light; and the lack of a proper 'Krebs cycle' as a result of down-regulated steps and alternative pathways. In summary, carbon sources for day respiratory decarboxylations are chloroplastic pyruvate, pyruvate (and organic acids formed therefrom) synthesized from triose phosphates exported by the chloroplast, and remobilized organic acids. 2OG, 2-oxoglutarate; GDC/SHMT, glycine decarboxylase/serine hydroxymethyl tranferase; GS/GOGAT, glutamine synthetase/glutamine oxoglutarate aminotransferase; I(C)DH, isocitrate dehydrogenase; HP, hydroxypyruvate; ME, malic enzyme; OAA, oxaloacetate; PDH, pyruvate dehydrogenase complex; PEP, phospho*eno*lpyruvate; PGA, 3-phosphoglyceric acid; P-GL, 2-phosphoglycolate; PK, pyruvate kinase; PPDK, pyruvate Pi dikinase; PPP, pentose phosphate pathway.

Box 1

Day respiration is experimentally difficult to access, simply because it represents a small CO_2 flux as compared with carboxylation (v_c) or photorespiratory release ($\Phi = v_o/2$). Several techniques have been implemented for decades, using classical gas exchange, fluorescence or isotopes. It should be noted that none of these techniques is perfect – they all have possible drawbacks.

Name and references	Principle	Advantages	Disadvantages
Kok method (Kok, 1948, 1949)	*A*/light curve and extrapolation of *A* at zero light using points above the break point	Very simple to carry out and can be done at the CO_2 mole fraction of interest	• Requires monitoring of very small flux near the light compensation point • The Kok effect is only loosely related to respiration and is rather caused by an increase in c_c and ΦPSII at low light
Laisk method (Laisk, 1977)	A/c_i curves at different light intensities. The common intersection point gives R_d as it is a constant in the equation: $A = v_c(1-\Gamma^*/c_c)-R_d$	Also provides an estimate of the compensation point in the absence of day respiration (Γ*)	• Requires monitoring of very small flux near the CO_2 compensation point • The common intersection point is sometimes not visible (triangular area) • Assumes R_d does not depend on CO_2 • Affected by internal conductance and refixation when performed with A/c_i curves instead of A/c_c curves
Cornic method (Cornic, 1973)	Uses the difference in CO_2 production under CO_2-free air in 21% (L_O), 0% O_2 (L_N) in the light or in darkness (p): $R_d = L_O - L_N - p + R_n$	Also provides an estimate of the photorespiratory efflux	• Requires CO_2-free air, which is far from physiological conditions • Requires O_2-free air, which impacts on respiration (anoxic effect)
Loreto method (Loreto *et al.*, 2001; Busch, 2013)	$^{12}CO_2$ from day respiration is monitored in a $^{13}CO_2$ atmosphere	Does not make any assumption on the expression of *A* and measures directly an efflux	• Relatively expensive (pure $^{13}CO_2$) • Assumes that respiratory substrates are not ^{13}C-labelled and this may be incorrect (e.g. chloroplastic decarboxylations)
Parnik method (Pärnik & Keerberg, 2007)	Radiometric mass balance of ^{14}C fixation and evolution	Provides estimates of respiration from stored and current photosynthates	• Manipulates radioactivity • Requires measurements at super-high CO_2 (3%) and low O_2
Gong method (Gong *et al.*, 2015)	Close to Loreto method, but uses CO_2 sources at natural ^{13}C abundance	Δ_A is measured, thus internal conductance can also be calculated	• Assumes that respiratory substrates are not $^{12}C/^{13}C$-labelled and this may be incorrect (e.g. chloroplastic decarboxylations)
Yin method (Yin *et al.*, 2011)	Close to the Kok method. Exploits the relationship between *A*, ΦPSII and irradiance along a light curve under nonphotorespiratory conditions: $A = S \times$ iPAR $\times$ ΦPSII$-R_d$, where *S* is a coefficient	Simple method to implement with fluorescence	• Requires monitoring of very small flux near the light compensation point • In principle, does not work well under photorespiratory conditions because *S* depends on c_c and Γ*, and thus is not constant along the curve

efflux in a CO_2-free air (Cornic, 1973), or ^{14}C labelling (Pärnik & Keerberg, 2007) yield mostly consistent results (see Table 1 for the list of symbols). That is, CO_2 evolution measured in the light appears to be lower than that in the dark. There is presently little evidence for a diel regulation of respiration at the transcription level (Rasmusson & Escobar, 2007; Florian *et al.*, 2014). Subtle reductions in the abundance of TCAP enzymes (citrate synthase, aconitase, NADP-dependent isocitrate dehydrogenase) have been observed in the *Arabidopsis* mitochondrial proteome of shoots in the light compared with the dark (Lee *et al.*, 2010). Causes of this inhibition are believed to be enzymatic (posttranslational or biochemical). Fructose-6-phosphate entry into glycolysis (phosphorylation to fructose-1,6-bisphosphate) is inhibited by the high triose phosphates : P_i ratio in the cytosol and the concurrent decrease in the metabolic effector fructose-2,6-bisphosphate (Stitt, 1990; Plaxton & Podestá, 2006). Furthermore, in the unicellular alga *Selenastrum minutum* and in tobacco (*Nicotiana tabacum*), total pyruvate kinase activity is lower in the light than in the dark (Lin *et al.*, 1989; Scheible *et al.*, 2000). The mitochondrial pyruvate dehydrogenase complex (PDH) (as opposed to the chloroplastic complex, which is not phosphorylatable) is also partly inactivated by (reversible) phosphorylation in illuminated leaves (Budde & Randall, 1990; Tovar-Mendez *et al.*, 2003). It is worth noting that the PDH is phosphorylated by a protein kinase, which is stimulated by conditions created by photorespiratory metabolism (e.g. NH_3, high ATP levels). *In vivo* ^{13}C-labelling has further shown that the PDH activity is inhibited by *c.* 30% in the light (Tcherkez *et al.*, 2005, 2008). Also, enzymes of the TCAP are partly inhibited in the light (Gessler *et al.*, 2009), probably because of the high mitochondrial NADH/NAD^+ (and ATP/ADP) ratio resulting from photorespiratory glycine decarboxylation (Gardeström & Wigge, 1988; Hurry *et al.*, 2005). It has also

Table 1 List of symbols

Symbol	Units commonly used	Definition
A	μmol m^{-2} s^{-1}	CO_2 net assimilation
c_a	μmol mol^{-1}	Atmospheric CO_2 mole fraction
c_c	μmol mol^{-1}	Stromal CO_2 mole fraction
c_i	μmol mol^{-1}	Intercellular CO_2 mole fraction
c_y	μmol mol^{-1}	Cytosolic CO_2 mole fraction
CUE	dl	Carbon-use efficiency
Δ_A	‰	$^{12}C/^{13}C$ isotope fractionation associated with net photosynthesis
Δ_P	‰	$^{12}C/^{13}C$ isotope fractionation associated with net photosynthesis in the absence of day respiration
e	‰	$^{12}C/^{13}C$ isotope fractionation associated with day respiration
Φ	μmol m^{-2} s^{-1}	Total photorespiratory CO_2 efflux
Φ_c	μmol m^{-2} s^{-1}	Photorespiratory CO_2 flux that diffuses to the chloroplast
Φ_y	μmol m^{-2} s^{-1}	Photorespiratory CO_2 flux that diffuses to the cytosol
g_s	mol m^{-2} s^{-1}	Stomatal conductance for CO_2 diffusion
$g_{m\ app}$	mol m^{-2} s^{-1}	Apparent internal conductance ($= A/(c_i - c_c)$)
g_{mw}	mol m^{-2} s^{-1}	Conductance for CO_2 dissolution and diffusion through cell wall and plasma membrane
g_{mc}	mol m^{-2} s^{-1}	Conductance for CO_2 diffusion through chloroplastic envelope
Γ^*	μmol mol^{-1}	CO_2 compensation point in the absence of day respiration
k	mol m^{-2} s^{-1}	Carboxylation efficiency ($= v_c/c_c$)
NEP	μmol m^{-2} s^{-1}	Net ecosystem CO_2 exchange
R_d	μmol m^{-2} s^{-1}	Total day respiratory CO_2 efflux
R_{dc}	μmol m^{-2} s^{-1}	Respiratory CO_2 flux that diffuses from the mitochondrion to the chloroplast
R_{dd}	μmol m^{-2} s^{-1}	Respiratory CO_2 flux associated with cytosolic decarboxylations (e.g. PPP)
R_{dh}	μmol m^{-2} s^{-1}	Respiratory CO_2 flux associated with leaf heterotrophic cells
R_{dp}	μmol m^{-2} s^{-1}	Respiratory CO_2 flux associated with chloroplastic decarboxylations (e.g. mPDH)
R_{dy}	μmol m^{-2} s^{-1}	Respiratory CO_2 flux that diffuses from the mitochondrion to the cytosol
R_n	μmol m^{-2} s^{-1}	Respiration of dark-adapted leaves (night respiration)
v_c	μmol m^{-2} s^{-1}	Rubisco-catalysed carboxylation rate
v_o	μmol m^{-2} s^{-1}	Rubisco-catalysed oxygenation rate

been shown that activity of mitochondrial isocitrate dehydrogenase is inhibited by high NAD(P)H/NAD(P)$^+$ ratios that may occur in the light (Igamberdiev & Gardeström, 2003; but see Kasimova *et al.*, 2006).

2. Carbon allocation within the TCAP in the light

The reduced TCAP activity in the light has been monitored via ^{13}C-labelling of detached leaves of French bean (*Phaseolus vulgaris*): when supplied with ^{13}C-1-pyruvate, $^{13}CO_2$ was produced in the light. As other pyruvate-decarboxylating metabolic pathways (such as the 2-C-methyl-erythritol-4-phosphate (MEP) dependent isoprenoid biosynthesis) are typically associated with a small flux, this mostly reflects the *in vivo* activity of the PDH. However, when supplied with ^{13}C-3-pyruvate, ^{13}C-labelling of both day-respired CO_2 and citrate was very modest, showing the weak activity of the malic enzyme and enzymes of the TCAP (Tcherkez *et al.*, 2005). O_2-consumption measurements with isolated mitochondria extracted from illuminated spinach leaves (*Spinacia oleracea*) and supplied with either exogenous malate, succinate or citrate showed that citrate gives the lowest respiration rate; in addition, when malate was supplied, it was mainly converted to citrate and pyruvate, with < 1% of isocitrate or fumarate (Hanning & Heldt, 1993). Using deuterium (^2H) enrichment and isotopic labelling with either $^{13}CO_2$ or ^{13}C-pyruvate, it has been shown that the commitment of ^{13}C-atoms to TCAP-associated decarboxylations was very limited in illuminated leaves of cocklebur (*Xanthium strumarium*), with citrate synthase being a possible limiting step (Tcherkez *et al.*, 2009). A recent analysis of ^{13}C-content in amino acids (alanine, glutamate and aspartate) after $^{13}CO_2$ labelling has provided evidence that the commitment of current photosynthates to the TCAP is in the order of 0.02–0.05 μmol m^{-2} s^{-1} across different species, and that the contribution of reserve remobilization to feed the TCAP varies between 20% and 80% depending on gaseous (CO_2, O_2) conditions (Abadie *et al.*, 2017b).

3. Pyruvate metabolism in the light

The above overview shows the limited capacity of pyruvate molecules to enter the TCAP in the light, arguably by inhibition of mitochondrial PDH. This raises a question about the fate of pyruvate and acetyl-CoA molecules in the mitochondrion. Acetyl-CoA is not likely to accumulate. First, PDH is end-product-inhibited, by acetyl-CoA (Harding *et al.*, 1970; Miernyk *et al.*, 1987; Rapp *et al.*, 1987). Second, a significant fraction of acetyl-CoA is directed to fatty acid production in the chloroplast (Ohlrogge & Jaworski, 2003). Accordingly, the mutant line of *Arabidopsis* that produces antisense RNA of the PDH kinase (thus enhancing the mitochondrial PDH reaction) accumulated ^{14}C-labelled fatty acids when ^{14}C-Pyr was fed to photosynthetic stems (Marillia *et al.*, 2003). Potentially, pyruvate can simply accumulate or be consumed by major reactions other than PDH-catalysed

dehydrogenation: the reverse reaction of pyruvate kinase, utilization by pyruvate P_i dikinase (both evolving phospho*enol*pyruvate, PEP), or amination to alanine by alanine aminotransferase.

Metabolomics analysis of leaves during a day–night cycle have shown that the pyruvate content is roughly twofold larger in the light (Scheible *et al.*, 2000). Moreover, pyruvate has been shown to yield alanine, as shown by ^{13}C-labelling (Tcherkez *et al.*, 2005). Double isotopic pyruvate tracing using ^{13}C and ^{2}H has also shown that pyruvate can reform PEP via pyruvate P_i dikinase (Tcherkez *et al.*, 2011a) and this enzyme is more active in the light than in the dark (Chastain *et al.*, 2002). Consumption of pyruvate by pyruvate kinase during the reverse reaction, generating PEP, is highly unlikely considering the equilibrium constant of the reaction (Tcherkez *et al.*, 2011a). As stated earlier, pyruvate production by pyruvate kinase is inhibited in the light, owing to the regulatory properties of the enzyme. In effect, in tobacco leaves, the total activity of pyruvate kinase has been shown to be lower in the light than in the dark (Scheible *et al.*, 2000). The algal enzyme (from *S. minutum*) is inhibited by photosynthetic intermediates (e.g. ribulose 1,5-bisphosphate) and the cytosolic enzyme is inhibited by P_i and glutamate (Lin *et al.*, 1989). Furthermore, leaf pyruvate kinases are inhibited by citrate (Baysdorfer & Bassham, 1984). Consequently, in the light, pyruvate kinase activity is probably down-regulated in the chloroplast and the cytoplasm, where it is adjusted by the balance between upstream and downstream metabolites (Fig. 1). Recently, a double 'omics' analysis (phosphoproteomics and metabolomics) in *Arabidopsis* has demonstrated a concerted phosphorylation pattern in PEP carboxylase (PEPC), PDH and pyruvate Pi dikinase, with resulting changes in pyruvate, alanine, γ-aminobutyrate and citramalate content (Abadie *et al.*, 2016b). These results confirm the key role of protein phosphorylation in pyruvate metabolism regulation in the light.

Taken as a whole, day respiratory metabolism is associated with a reorchestration of major pathways (glycolysis and pyruvate metabolism), resulting in lower CO_2 efflux as compared with darkness. However, a comprehensive analysis of metabolic fluxes, including reserves remobilization and partitioning at branching points, is still lacking so that the specific origin of carbon atoms found in day-respired CO_2 (i.e. proportions of CO_2 produced by PDH, TCAP, the pentose phosphate pathway and other reactions) is not known with certainty.

IV. Significance of day respiration for leaf N assimilation

In illuminated leaves, nitrogen reduction and assimilation involves nitrate and nitrite reductase and the glutamine synthetase/glutamine 2-oxoglutarate amino transferase (GS/GOGAT) cycle that yields glutamate (for a review, see Forde & Lea, 2007). Regulation of the activities of these enzymes and requirements for ATP and reductants are such that nitrogen is assimilated in leaves mostly in the light, as compared with the dark (Delhon *et al.*, 1995; Stitt *et al.*, 2002). Nitrogen sources and their relationships to nitrogen metabolism were first documented nearly 40 yr ago. While roots are responsible for a variable, species-specific proportion of nitrate reduction in either the dark or the light (Radin, 1978), ^{15}N-isotopic labelling has shown that nitrate molecules not consumed by roots in darkness are exported to shoots, where they accumulate and become available for reduction during the subsequent light period (Gojon *et al.*, 1986). Although leaf nitrate content is often large, thereby enhancing isotopic dilution and impeding ^{15}N labelling, nitrogen recycling (e.g. protein hydrolysis) in leaf cells is evidenced by the inability to completely label glutamate with ^{15}N (Bauer *et al.*, 1977).

1. Steps of 2-oxoglutarate production in the light

The source of carbon used during nitrogen assimilation ultimately comes from respiration, 2-oxoglutarate (2OG) being the carbon skeleton required to run the GS/GOGAT cycle. Within plant cells, 2OG is mostly generated by isocitrate dehydrogenases. There are several isoforms, NAD- or NADP-dependent (enzymes are abbreviated IDH and ICDH, respectively), and ICDH enzymatic activity is present in different cell compartments (Gálvez *et al.*, 1999; Hodges, 2002). Isocitrate dehydrogenation represents one step of the TCAP. However, the metabolic origin of 2OG is not clear, because in *Arabidopsis*, mutants affected in I(C)DH (*icdh 2* and *idh V*) lack a strong phenotype and seem to grow normally. In fact, knockdown mutations of cytosolic ICDH lead to little metabolic effect (the majority of metabolic pools are affected by < 1.5-fold, except for glutathione and cysteine; Mhamdi *et al.*, 2010) and, similarly, knockdown mutations of IDH caused variable and mostly insignificant changes in metabolite pools, although several TCAP intermediates accumulated under heterotrophic liquid culture conditions (Lemaitre *et al.*, 2007). In IDH antisense tomato (*Solanum lycopersicum*) lines, the 2OG : glutamate ratio is increased but there was little effect on organic and amino acid content, despite a slightly lower labelling in TCAP intermediates upon ^{13}C-pyruvate feeding (Sienkiewicz-Porzucek *et al.*, 2010). From consideration of respiration rates and IDH activity, calculated control coefficients are small for IDH (Araujo *et al.*, 2012). It seems likely that the involvement of several I(C)DH isoforms compensates for each individual mutation described earlier. In addition, isotopic tracing using I(C)DH sesquimutants (i.e. *icdh* $2^{+/-}$ *idh* $V^{-/-}$ and *icdh* $2^{-/-}$ *idh* $V^{+/-}$, with considerable reduction in total I(C)DH activity) has shown that lysine synthesis (from aspartate) and degradation can serve as an alternative pathway for 2OG generation in leaves (Boex-Fontvieille *et al.*, 2013).

2. Reserve remobilization

Metabolic pathways of 2OG production for N assimilation in the light remain uncertain. On the one hand, the day respiration rate R_d has been shown to be sensitive to N assimilation (Guo *et al.*, 2005), suggesting that it may provide some of the necessary 2OG. In addition, calculations based on stored leaf citrate content available at the beginning of the light period suggest that it is insufficient to support 2OG synthesis for glutamate production (Stitt *et al.*, 2002), and thus day respiration might be critical for 2OG synthesis. This process is accompanied by anapleurotic activity of PEPC (Huppe & Turpin, 1994), producing oxaloacetate that can be

either used by citrate synthase or transaminated to aspartate. On the other hand, the remobilization of substrates produced in darkness certainly plays a role, because it might supply carbon skeletons without requiring all of the steps of the TCAP in the light. However, mutants affected in either aconitase or isocitrate dehydrogenase activity do not show clear reductions in plant biomass or N content (Kruse *et al.*, 1998; Carrari *et al.*, 2003; Lemaitre *et al.*, 2007). Presumably, therefore, the carbon source for glutamate production includes both newly synthesized (TCAP-derived) and remobilized (from night-accumulated organic acids) 2OG. Even so, the proportion derived from remobilization seems to be larger than that from *de novo* synthesis. Double isotopic labelling ($^{13}CO_2$, ^{15}N-ammonium nitrate) and examination of ^{13}C-^{15}N spin–spin interactions have shown that most of the assimilated ^{15}N is fixed onto remobilized (non-^{13}C-labelled) substrates (i.e. the proportion of ^{13}C substrates utilization in total ^{15}N-fixation is small) and, conversely, *c.* 50% of the visible ^{13}C-amino acids are ^{15}N-labelled, showing that N assimilation is an important fate of neosynthesized 2OG; in addition, returning to a $^{12}CO_2$ atmosphere after a period of darkness shows a ^{13}C-enrichment in citrate, glutamine and glutamate, clearly demonstrating the recycling of previously fixed carbon atoms (Gauthier *et al.*, 2010). Accordingly, CO_2 decarboxylated by day respiration has been shown to comprise a substantial part of 'old' remobilized carbon. Similarly, ^{13}C-labelling and mass balance calculations have demonstrated the key role of remobilization to synthesize glutamate in the light (Abadie *et al.*, 2017b). ^{14}C-labelling and radiometric studies of day-evolved CO_2 have suggested that up to 40% of decarboxylated CO_2 comes from stored, slowly turned-over carbon molecules (Pärnik *et al.*, 2002; Pärnik & Keerberg, 2007). The isotopic disequilibrium (at ^{13}C natural abundance) between current photosynthates and day-respired CO_2 has also suggested that day respiration utilizes remobilized substrates (Wingate *et al.*, 2007; Tcherkez *et al.*, 2010, 2011b, 2012).

3. Overall N-flux and anapleurotic activity

The metabolic mechanisms by which remobilized substrates are recycled are not obvious, as the recycling of malate, fumarate or citrate (the most common accumulated organic acids in C_3 plants) would require the action of citrate synthase and/or isocitrate dehydrogenase, two steps that are assumed to be partly inhibited in the illuminated leaf (see earlier and Fig. 1). It is plausible that very small fluxes through the TCAP are sufficient to meet N assimilation requirements in addition to alternative (as yet unknown) pathways. As a matter of fact, calculation of the presumed average flux required for N assimilation (*c.* 0.05 μmol m^{-2} s^{-1}) is close to that measured through the TCAP in illuminated leaves (Tcherkez & Hodges, 2008). The production of TCAP intermediates is supplemented by PEPC activity in the light (often assumed to be 5% of the net assimilation rate in C_3 plants, i.e. near 0.5 μmol m^{-2} s^{-1}). PEPC can compensate for the consumption of organic acids (such as 2OG) by N assimilation, by providing oxaloacetate (malate) to feed the TCAP (the so-called anapleurotic function of PEPC). Further, some oxaloacetate molecules can be directly aminated to aspartate (Huppe & Turpin, 1994). This relationship between PEPC and aspartate metabolism has been evidenced by a consistent body of experimental data, and was reviewed in Tcherkez & Hodges (2008).

In summary, the TCAP does not maintain its cyclic nature in illuminated leaves (Tcherkez *et al.*, 2009; Abadie *et al.*, 2017b), and a substantial portion of 2OG molecules are consumed for N assimilation to glutamate, while the PEPC activity maintains aspartate pools as well as those of malate and fumarate through backward reactions of the reversible enzymes malate dehydrogenase and fumarase (Fig. 1).

V. Significance of day respiration for leaf gas exchange

Under standard conditions, that is, at 400 μmol mol^{-1} CO_2 and 21% O_2 and 20–25°C, the day respiration rate (R_d) represents *c.* 5% of net assimilation in leaves with high photosynthesis and can be much higher in plants with low photosynthesis rates (Atkin *et al.*, 2000). Therefore, the impact in terms of carbon balance may appear relatively modest at first glance (but see section VII). The rate R_d and the ratio R_d/A nevertheless vary considerably depending on species, environmental conditions such as CO_2 (see, e.g. Kroner & Way, 2016) or N nutrition (Guo *et al.*, 2005).

1. Experimental determination of R_d and the problem of CO_2 refixation

An earlier discussion in this review points to the fact that R_d cannot be simply predicted from A, and must instead be experimentally determined. However, measuring R_d accurately is a persistent, nontrivial problem. Methods for this have been reviewed (Tcherkez & Ribas-Carbó, 2012) and some other techniques have been proposed recently, based on fluorescence (Yin *et al.*, 2014), and ^{12}C/^{13}C isotopic disequilibrium (Gong *et al.*, 2015, 2017) (Box 1). Amongst this range of techniques, the Kok method (photosynthesis response curve at very low light) may not be the best adapted to measure R_d because of additional, confounding factors like possible changes in c_c (Farquhar & Busch, 2017; but see Buckley *et al.*, 2017) or photochemical efficiency (Tcherkez *et al.*, 2017). The Laisk method (photosynthetic response curves at low CO_2 at multiple irradiances) is also potentially problematic considering the response of day respiration to CO_2 mole fraction (see the next section). The method that exploits respiratory $^{12}CO_2$ release in a $^{13}CO_2$ atmosphere provides a more 'natural' way to measure R_d because it does not require changing either the CO_2 mole fraction or light. Using this method, the inhibition of leaf respiration by light has been confirmed ($R_d < R_n$, where R_n stands for respiration in darkness) and suggested to be partly a result of refixation (Loreto *et al.*, 2001; Pinelli & Loreto, 2003).

At this stage, it is important to emphasize that refixation is already taken into account in classical equations describing net photosynthesis based on c_c (Box 2), which should be used with the Laisk method. That is, in principle, the fact that R_d is consistently found to be lower than R_n cannot be caused by refixation. However, the Laisk method is commonly implemented using A/c_i curves

Box 2

Leaf gas exchange usually models day respiration as a constant flux of CO_2, usually denoted as R_d. Therefore, if net CO_2 assimilation is denoted as A and photorespiratory CO_2 release as Φ, it gives:

$$A = v_c - \Phi - R_d \quad \text{Eqn B1}$$

As such, R_d does not depend on CO_2 nor O_2 (and thus does not depend on internal CO_2 mole fraction, c_c), although recent experimental evidence on metabolism seems to suggest the contrary (see text). It is worth noting that this equation integrates the possibility that CO_2 evolved by day respiration might be refixed by photosynthesis. In fact, this equation comes from the steady-state hypothesis on c_i, the intercellular CO_2 mole fraction. A is the net influx of CO_2 through stomatas and can be written as $g_s(c_a - c_i)$ so that c_i is effectively the net result of gross influx ($g_s c_a$), outflux (retrodiffusion, $g_s c_i$), gross fixation (v_c), photorespiration (Φ) and day respiration (R_d). A similar argument can be articulated with c_c if day-respired CO_2 is assumed to be liberated internally. Furthermore, Eqn B1 remains valid even if cellular distribution of CO_2 pools differentiates cytoplasmic and chloroplastic compartments (see Fig. 2). Similarly, in equations that describe the $^{12}C/^{13}C$ isotope fractionation during net photosynthesis (Δ), refixation of day respiration is taken into account (steady state on the isotope composition of internal CO_2). Using the expression of Φ (as a function of v_c), Eqn B1 gives:

$$v_c = \frac{A + R_d}{c_c - \Gamma^*} \quad \text{Eqn B2}$$

Eqn B2 is seminal for computations of carboxylation rates, or c_c (or electron flux J_c) with fluorescence, for example. When A is large, potential errors caused by variation in R_d are small. However, when A is relatively small, at low light or low CO_2 (or high O_2), errors in v_c can be quite substantial. Also, it should be noted that R_d is a term that represents a sum of CO_2 evolved by photosynthetic and heterotrophic cells, from different cell compartments, using reserve remobilization or net fixed carbon from current photosynthesis. Although this does not complicate Eqns B1 and B2, it has important consequences for the equation describing the $^{12}C/^{13}C$ fractionation (see text), and the expression of apparent internal conductance $g_{m\ app}$. The decomposition into subcellular fluxes, as depicted in Fig. 2, leads to:

$$g_{m\ app} = \left(\frac{1 + \varepsilon'}{g_{mw}} + \frac{1 + \varepsilon}{g_{mc}} \right)^{-1} \quad \text{Eqn B3}$$

where $\varepsilon = (\Phi_y + R_{dy} + R_{dd})/A$ (relative amount of (photo)respired CO_2 escaping the mitochondrion + cytosolic CO_2 production) and $\varepsilon' = R_{dh}/A$ (relative amount of heterotrophic CO_2). This is illustrated in Fig. B1 which shows that varying ε and ε' can cause some variation (up to 20%) in computed apparent internal conductance.

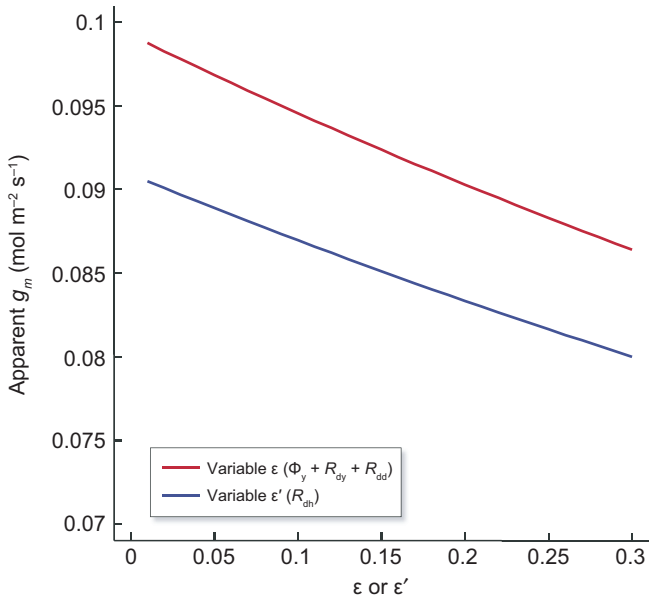

Fig. B1 Impact of varying the amount (denoted as ε) of (photo)respired CO_2 that escapes the mitochondrion to the cytoplasm, i.e. impacting c_y (red) or that of leaf heterotrophic respired CO_2 (from parenchyma, phloem cells, etc.; denoted as ε') liberated in intercellular spaces, that is, impacting c_i (blue). In this numerical example, A is fixed at 10 μmol m^{-2} s^{-1}, and stomatal, wall and chloroplast envelope conductances at 0.25, 0.2 and 0.2 mol m^{-2} s^{-1}, respectively, and Eqn B3 is used.

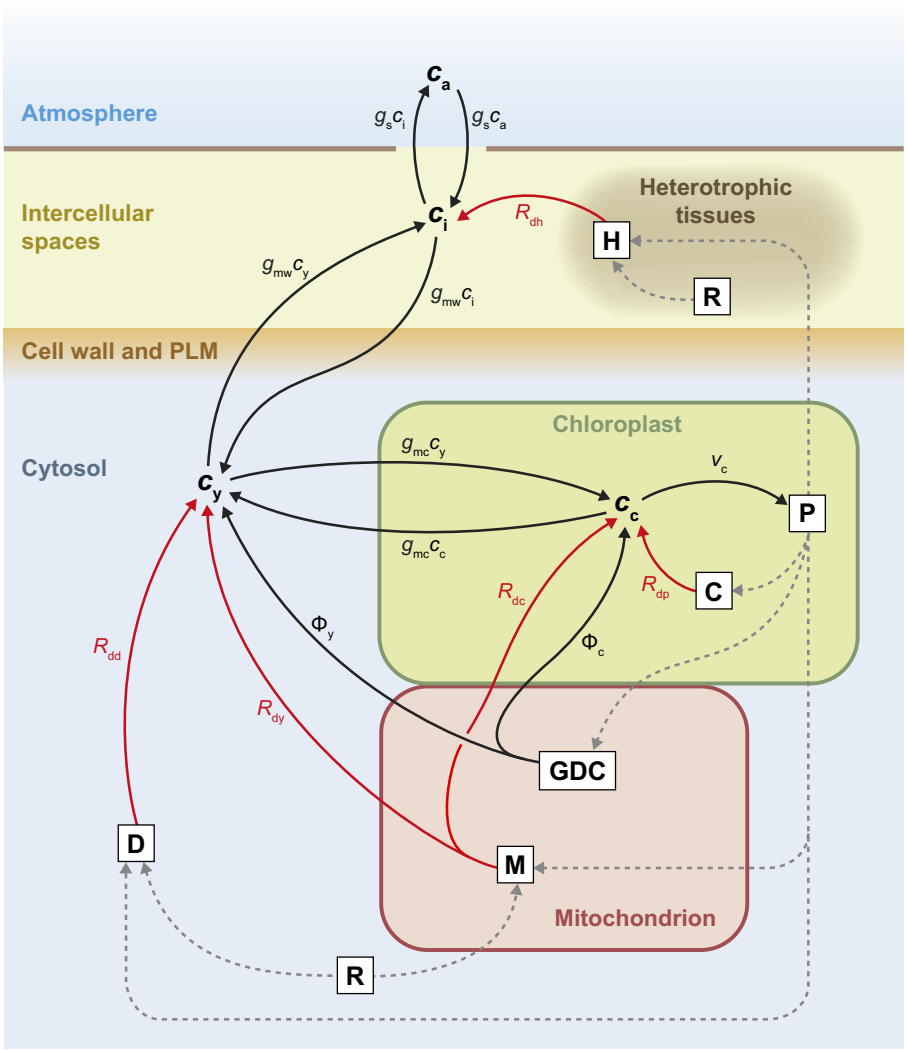

Fig. 2 CO_2 flux model accounting for the subdivision of day respiration into components (in red): cytoplasmic decarboxylations like the PPP or cICDH (R_{dd}); chloroplastic decarboxylation catalysed by cPDH (R_{dp}); mitochondrial metabolism (mPDH and TCAP) evolving CO_2 which may directly diffuse to the chloroplast as a result of the close physical association of the two organelles (R_{dc}) in mesophyll cells, or escape the mitochondrion to the cytosol (R_{dy}); respiration by heterotrophic tissues of the leaf (R_{dh}). Thus, total day respiration is given by $R_d = R_{dd} + R_{dp} + R_{dc} + R_{dy} + R_{dh}$. Photorespiratory CO_2 may also directly diffuse back to the chloroplast (Φ_c) or escape to the cytosol (Φ_y) (thus total photorespiratory CO_2 production is $v_o/2 = \Phi_y + \Phi_c$). Internal conductance is here subdivided into two elemental steps: cell wall and plasma membrane (g_{mw}) and chloroplastic envelope (g_{mc}). Stomatal conductance is denoted as g_s. Framed letters stand for simplified metabolic pathways: C, chloroplastic catabolism; D, cytosolic decarboxylations; GDC, photorespiratory glycine decarboxylation; H, metabolism of heterotrophic tissues; M, mitochondrial metabolism (mPDH + TCAP); P, photosynthates and phosphorylated intermediates; R, remobilization of reserves. Note the possible utilization of both reserves and current photosynthates to feed respiration (except for cPDH, the substrate of which – pyruvate – is most probably synthesized directly by chloroplastic glycolysis, from triose phosphates produced by current photosynthesis). The CO_2 mole fraction is denoted as c_a (atmosphere), c_i (intercellular spaces), c_y (cytosol) and c_c (chloroplasts, carboxylation site). v_c is the carboxylation rate by Rubisco. For clarity, this figure does not show PEPC-catalysed carboxylation and equilibria between dissolved CO_2 and HCO_3^-.

instead of A/c_c curves and, in that case, it neglects the effect of internal conductance and thus the possible refixation of internal CO_2. Also, refixation cannot be the cause of R_d being lower than R_n using the Kok method: typically, in 2% O_2, the Kok effect disappears, meaning that the apparent inhibition of respiration by light also disappears (reviewed in Tcherkez *et al.*, 2017). If refixation were the driver of the apparent inhibition of respiration, we would expect the opposite, because carboxylation, and thus refixation, is enhanced at low oxygen.

Determining the proportion of refixed CO_2 remains technically challenging. Recently, refixation was estimated using a change in atmospheric isotope composition from $^{12}CO_2$ to $^{13}CO_2$, and super-high concentration of $^{13}CO_2$ (10 000 μmol mol^{-1}) to prevent the reassimilation of $^{12}CO_2$ evolved from photorespiration and day respiration. In this way, it has been suggested that 46–59% of day-respired CO_2 was reassimilated (Busch *et al.*, 2013). Isotopic labelling with ^{13}C-enriched respiratory substrates under a standard atmosphere (380 μmol mol^{-1} CO_2, 21% O_2) and subsequent analysis of ^{13}C amount in starch have suggested that up to 15% of decarboxylated CO_2 was refixed (Tcherkez *et al.*, 2008; Tcherkez, 2013).

Presumably, the proportion of refixation is likely to depend on physiological and/or anatomical conditions that determine internal

conductances, such as that of the chloroplast envelope or from intercellular to cytoplasmic compartments (Fig. 2), which might vary independently. That is, the fate of the CO_2 molecules produced by day respiration and by photorespiration (namely, immediate refixation or liberation in cytoplasmic CO_2) can change the calculation of apparent internal conductance defined by $g_m = A/(c_i - c_c)$, or total conductance defined by $g_t = A/(c_a - c_c)$. In fact, g_m is given by $((1+\varepsilon')/g_{mw} + (1+\varepsilon)/g_{mc})^{-1}$ where g_{mw} and g_{mc} are the conductances associated with cell wall and chloroplast envelope, respectively (for calculations, see Tholen *et al.*, 2012; Tcherkez, 2013; Farquhar & Busch, 2017; and Box 2); ε is the ratio of (photo) respired CO_2 liberated in the cytosol (plus cytoplasmic decarboxylation) to A; and ε' is the ratio of heterotrophic respiration (parenchyma, phloem cells, etc.) to A. The value of ε is determined by the fate of CO_2 molecules within the cell and their opportunity to escape (i.e. diffuse out of) the leaf, as well as metabolic pathways in the cytosol.

In the case of day respiration (again, defined as nonphotorespiratory CO_2 evolution in the light) it should be kept in mind that extramitochondrial decarboxylations occur in the cytoplasm, such as that of the pentose phosphate pathway (6-phosphogluconate dehydrogenase) and cytoplasmic isocitrate dehydrogenase (ICDH, see above). Hence, ε is certainly greater than zero, so that measured internal conductance to CO_2 diffusion in leaves depends, in part, on the relative rates of the various extramitochondrial decarboxylations, which are influenced by metabolic flux control in catabolism. Also, the location of mitochondria and chloroplasts within the cytosol will influence refixation of respired CO_2, and thus estimates of CO_2 diffusional conductances, and this is further complicated by the movement of these organelles in response to environmental and physiological conditions. Similarly, heterotrophic respiration (flux denoted as R_{dh} in Fig. 2) can have an impact on apparent internal conductance. Unfortunately, up to now, the effect of day respiratory metabolic components on internal conductance has not been investigated.

It should also be noted that although CO_2 exchange represents the prevalent way in which leaf respiration is considered in the current literature, respiration is also an exchange of oxygen (O_2). Unsurprisingly, oxygen-based measurements of R_d are much more difficult as a result of the enormous background of 21% O_2. Performing experiments at low oxygen (e.g. 2%) may prevent this problem but they should be avoided as recent data have shown that leaf respiratory metabolism is significantly perturbed under such gaseous conditions, with typical symptoms of hypoxia (Tcherkez *et al.*, 2012; Abadie *et al.*, 2017a). Oxygen fluxes can also be deconvoluted using $^{16}O/^{18}O$ isotopes, but this requires expensive labelling and mass spectrometry (e.g. Peltier & Thibault, 1985). As a result, there is a lack of data on oxygen-based R_d values, and the respiratory quotient (CO_2/O_2) of day respiration is currently unknown.

2. Influence of R_d on carbon isotope exchange

The effect of day respiration on carbon balance is proportionally larger when assimilation is low, such as at low light or at low CO_2. Day respiratory CO_2 release also has a critical impact on the $^{12}C/^{13}C$ isotope fractionation associated with net photosynthesis (Δ_A), which is given by (neglecting ternary effects) (Farquhar *et al.*, 1989; Wingate *et al.*, 2007; Tcherkez *et al.*, 2010, 2011b; Gong *et al.*, 2015):

$$\Delta_A = \Delta_P - \frac{e_{cur} R_{cur}}{k c_a} - \frac{e_{dis} R_{dis}}{A} \quad \text{Eqn 1}$$

where Δ_P is the fractionation associated with photosynthesis in the absence of day respiration (i.e. diffusion, carboxylation and photorespiratory CO_2 release) (see also Table 1 for the list of symbols). R_{dis} is CO_2 evolution from stored carbon reserves disconnected from current photosynthesis. It should be noted that R_{dis} is not simply equal to heterotrophic respiration (R_{dh}) as carbon reserves may also sustain respiratory metabolism in mesophyll cells (Fig. 2). R_{cur} is CO_2 evolution from net fixed carbon (current photosynthates). e_{dis} and e_{cur} are the associated fractionation values, and k is carboxylation efficiency ($= v_c/c_c$, where v_c is carboxylation velocity). Eqn 1 shows that the isotopic difference between net fixed carbon and respiratory reserves (fractionation factor e_{dis}) is scaled by the respiration-to-assimilation ratio ($R_{dis} : A$) rather than respiration-to-carboxylation ratio ($R : v_c$) (Tcherkez *et al.*, 2011b; see also Box 2). The former tends to infinity at low A (at very low light or low CO_2), and this effect may be aggravated (with observed Δ_A as high as 100‰) when net fixed CO_2 is isotopically distinct from respiratory substrates. This situation typically occurs when inlet CO_2 used during gas exchange experiments is from a source different from that used to grow plants, and recent experiments showed this typical behaviour at low light or low CO_2, that is, close to photosynthetic compensation points (Barbour *et al.*, 2017).

Taken as a whole, day respiration is a minor component of leaf gas exchange under most conditions, regardless of the proportion of refixed CO_2. By contrast, it can affect substantially isotopic mass balance at low or modest assimilation rates, and has a nonnegligible impact on internal conductance calculations.

VI. Is day respiration influenced by CO_2 mole fraction?

The effects on plant carbon exchange of increases in atmospheric CO_2 concentration associated with industrialisation have attracted considerable research interest for many years. However, the influence of CO_2 mole fraction on R_d remains unclear, as a result of variability in the magnitude and even direction of observed responses. For example, the direction of response seems to be opposite in the short and long term.

1. Short-term effects of CO_2 mole fraction

In the short term, the effect of CO_2 mole fraction on day respiration is not very well known, partly because the Laisk method requires a response curve to CO_2 and thus assumes that R_d is independent of CO_2 mole fraction. Using the Kok method, CO_2 mole fraction has been found to have no effect on R_d (Sharp *et al.*, 1984; Tcherkez *et al.*, 2012; but see Evans, 1987; Björkman & Demmig, 1987, for super-high CO_2 effects). However, using the same method (Kok), a relationship has been found between $R_d : R_n$ (light-to-dark

respiration ratio) and the rate of oxygenation v_o (Griffin & Turnbull, 2013), suggesting that internal CO_2 (c_c) has an effect on R_d. Measurement using atmospheric $^{12}C/^{13}C$ isotope substitution suggests that R_d increases as the CO_2 mole fraction decreases (Pinelli & Loreto, 2003). Isotopic labelling of respiratory substrates and analysis of evolved CO_2 has been carried out in cocklebur leaves under different CO_2/O_2 conditions and an increase in decarboxylation reactions as photorespiration increases (including at low CO_2) has been found (Tcherkez *et al.*, 2008).

Isotopic tracing with ^{13}C-citrate has further shown that citrate metabolism decreases with CO_2 mole fraction (Tcherkez *et al.*, 2012). Interestingly, in the same study, the TCAP did not behave similarly at high CO_2 (800 μmol mol^{-1}, in 21% O_2) and 2% O_2 (at 400 μmol mol^{-1} CO_2), suggesting that low oxygen has specific effects on day respiration that are not comparable to those at high CO_2. Still, the effect of CO_2 on day respiratory metabolism suggests that c_c and thus the rate of photorespiration controls the TCAP. Amongst the key molecules involved in the TCAP is 2OG, which can be interconverted to glutamate via aminotransferases and the GS-GOGAT cycle. The relative commitment to 2OG has been found to increase as v_o/v_c increases, using isotopic labelling (Tcherkez *et al.*, 2012). The rationale of this effect is believed to be linked to the metabolic demand by photorespiration. In fact, photorespiratory metabolism comprises glutamate utilization (to generate glycine) and glutamate synthesis (recycling NH_3 via the GS/GOGAT cycle) (Fig. 1). Under the assumption that oxygenation changes abruptly or that photorespiratory reactions are not strictly quantitative, there could be a metabolic imbalance. This phenomenon has been suggested to occur on the basis of photosynthetic response curves (Harley & Sharkey, 1991). Also, the fact that glycine accumulates progressively in the light and that the glycine : serine ratio also tends to increase (see e.g. Novitskaya *et al.*, 2002) suggests that the conversion of glycine into serine by the glycine decarboxylase-serine hydroxymethyl transferase complex (GDC-SHMT) is not strictly quantitative. Direct assessment of glycine recycling efficiency in photorespiration has been undertaken recently using ^{15}N labelling and quantitative NMR analyses: in sunflower leaves, it has been shown that a small proportion of glycine molecules accumulates (*c.* 4% at 400 μmol mol^{-1} CO_2 and 21% O_2) and this effect is exaggerated at high photorespiration rates (low CO_2 or 100% O_2) (Abadie *et al.*, 2016a). These data are consistent with results obtained upon $^{15}N_2$-glutamine labelling of rapeseed (*Brassica napus*) leaves at 400 or 100 μmol mol^{-1} CO_2 (Gauthier *et al.*, 2010). ^{15}N analyses show a difference in ^{15}N allocation between ordinary and low CO_2 in favour of glycine at low CO_2. In addition, in both Abadie *et al.* (2016a) and Gauthier *et al.* (2010), ^{15}N-serine is detectable and represents a part of accumulated ^{15}N, suggesting that serine itself is also not quantitatively recycled.

Taken as a whole, the metabolic imbalance (accumulation of nonrecycled amino acids) has to be compensated for by supplemental nitrogen assimilation to sustain glutamate provision and thus glyoxylate conversion to glycine in photorespiration. Experiments using $^{14}CO_2$ have also shown that glutamate synthesis is promoted under photorespiratory conditions (Lawyer *et al.*, 1981): glutamate and glutamine represented a larger ^{14}C amount and glutamine had a higher ^{14}C-specific activity after $^{14}CO_2$-labelling in ordinary conditions as compared with nonphotorespiratory conditions. This is in agreement with the higher utilization of the electron flux to N reduction and assimilation observed at high photorespiration (Bloom *et al.*, 2002; Rachmilevitch *et al.*, 2004). Of course, the stoichiometric photorespiratory imbalance and thus the flux associated with the supplemental nitrogen assimilation is rather small: 4% of the usual oxygenation rate, that is, *c.* 0.1 μmol m^{-2} s^{-1} only. Nevertheless, this value is not negligible considering that day respiration (CO_2 efflux) is usually within the range 0.5–1.5 μmol m^{-2} s^{-1}. Taken as a whole, in the short term, CO_2 mole fraction impacts negatively on day respiration (i.e. with a stimulation at low CO_2 and a reduction at high CO_2), and this effect is probably driven partly by photorespiratory N metabolism.

2. Long-term effects of CO_2 mole fraction

In the long-term, the effect of CO_2 mole fraction on R_d has been shown to be quite variable. Growth at high CO_2 (730 μmol mol^{-1}) in cocklebur (*X. strumarium*) led to an increase in R_d of 30–50% (on leaf area basis, measured with the Kok method), while respiration in the dark changed less, so that the inhibition of respiration by light decreased (Wang *et al.*, 2001). By contrast, in Norway spruce (*Picea abies*), no significant effect of high CO_2 has been found on R_d (or R_n), regardless of temperature (Kroner & Way, 2016). Also in *Eucalyptus*, no significant CO_2 treatment effect has been found on R_d (Ayub *et al.*, 2011; Crous *et al.*, 2012, 2017). Herbaceous meadow species grown under a free air CO_2 enrichment experiment show no effect at all or a decrease in R_d (along with a decrease in R_n and variable resulting changes in the $R_d : R_n$ ratio) (Haworth *et al.*, 2015). Such variability is presumably a result of differences or concurrent changes in other environmental conditions such as soil quality, N availability, etc. as well as species differences. In fact, the net effect of changing CO_2 growth conditions on day (and night) respiration is the result of a complex interplay between photosynthetic input (increased sugar availability, see, e.g., Yelle *et al.*, 1989; Körner & Miglietta, 1994; Teng *et al.*, 2006), nitrogen availability and assimilation (Bloom *et al.*, 2014) and respiratory capacity, such as the amount of respiratory enzymes and the number of mitochondria (Griffin *et al.*, 2001; Wang *et al.*, 2004). For example, day respiration in cocklebur (measured with the Kok method) has been found to be influenced by the interaction between CO_2 and nitrogen availability (Shapiro *et al.*, 2004). It should also be noted that in the long term, units to express day and night respiration are important to consider, because leaf properties such as specific leaf area (g DW m^{-2}), N and S elemental content (%), etc. do change at high CO_2 (ordinarily, with an elemental dilution of N, i.e. a decline in %N). For example, R_d in soybean has been shown to decrease with growth CO_2 on a DW basis, but not on leaf area or nitrogen bases (Ayub *et al.*, 2014). Photosynthetic assimilation increases at high CO_2 so that the ratio $R_d : A$ (which is dimensionless) may not change (Ayub *et al.*, 2011; Kroner & Way, 2016). In wheat (*Triticum durum*) at the postanthesis stage, Aranjuelo *et al.* (2015) found that leaf night respiration increased under elevated CO_2 when expressed on a total protein (or N) rather than a leaf area basis, while the content in

TCAP enzymes also increased. Unfortunately, day respiration was not investigated in this study. In general, it has been found that genes associated with respiratory metabolism are up-regulated under high CO_2, suggesting a general increase in catabolism (Leakey *et al.*, 2009; Markelz *et al.*, 2014b) and this effect is partly suppressed under limiting N (Markelz *et al.*, 2014a). However, whether such effects cause a systematic stimulation of day respiratory metabolism under elevated CO_2 remains unknown.

At low CO_2, no significant effect on R_d (measured with the Kok method) has been found on a surface area basis in soybean (*Glycine max*; grown at 290 μmol mol^{-1} CO_2), while assimilation decreased significantly – leading to a higher R_d : A ratio – and the R_d : R_n ratio increased (Ayub *et al.*, 2014). In the mitochondrial complex I mutant CMS II of forest tobacco (*Nicotiana sylvestris*), which has a constitutively lower internal CO_2 mole fraction (c_c) (by *c.* 60 μmol mol^{-1}) due to low mesophyll conductance, R_d (measured with the Laisk method) is also found to be similar to that in the wild-type (Priault *et al.*, 2006).

In summary, long-term exposure to low CO_2 does not seem to have a significant effect on day respiratory efflux. However, day respiratory metabolism is considerably affected by CO_2 mole fraction, as a result of altered interactions with photorespiration and concurrent changes in nitrogen assimilation.

VII. Significance of day respiration at the plant and ecosystem levels

At the whole plant level, the impact of leaf day respiration on the carbon budget has to be accounted for in addition to respiratory losses by leaf night respiration and respiration of heterotrophic organs. Respiration thus plays a role in carbon-use efficiency (CUE), which is computed as:

$$\mathrm{CUE} = \frac{\text{Net primary production}}{\text{Gross primary production}} \qquad \text{Eqn 2}$$

that is, the ratio of net carbon gain accounting for carbon losses (integrated respiration) to assimilated carbon (integrated assimilation). Eqn 2 can be rewritten using the expression A ($= v_c - \Phi - R_d$), by taking into account respiratory losses in the light (R_d), as (Gifford, 2003):

$$\mathrm{CUE} = \frac{\int A - \int R_n}{\int A + \int R_d} \qquad \text{Eqn 3}$$

where R_n (night respiration) and R_d (day respiration) here integrate all plant organs.

One outcome of Eqn 3 is that the proportion represented by leaf respiration in the terms R_d and R_n may vary, depending on biomass distribution between plant organs, specific rates of respiration in different organs, and how these factors vary amongst plant species and growth conditions. Analysis of elemental C content and measurement of dark respiration in plants cultivated under high CO_2 have been used to show that even minimal changes in leaf respiration may have a significant impact on plant C budget (in other words, CUE is sensitive to leaf respiration rates) (Poorter *et al.*, 1992). More generally, not taking into account the inhibition of leaf respiration by light (i.e. the difference between R_d and R_n) may lead to significant overestimation of ecosystem respiration and thus an underestimation of CUE (for a recent review, see Heskel *et al.*, 2013). In fact, the use of a modified Kok method (despite the aforementioned inherent problems) (Bruhn *et al.*, 2011), isotopic mass balance (Wehr *et al.*, 2016) or empirical relationship between day respiration and light intensity (Wohlfahrt *et al.*, 2005) indicate that at ecosystem level, the light inhibition of R_d is high enough to significantly affect calculated daytime ecosystem gross CO_2 efflux.

That said, the impact of the inhibition of leaf respiration by light depends on plant species and ecosystems of interest (including environmental conditions such as temperature, and nutrient and water availability). For example, in a multisite study of European grasslands, a very good 1 : 1 correlation was found between fitted ecosystem day respiration (obtained from NEP/light curves, where NEP is net ecosystem CO_2 exchange) and night respiration rates (Gilmanov *et al.*, 2007). Using the same principle (NEP/light curves) in a North American forest, ecosystem respiratory efflux in the light was found to represent *c.* 60% of that during the night, on average (with considerable variation depending on temperature), suggesting a considerable impact of the inhibition of leaf respiration by light (Jassal *et al.*, 2007). In Arctic tundra plants, where assimilation rates are low (typically < 10 μmol m^{-2} s^{-1}), leaf R_d is proportionally large and changes in R_d driven by temperature may have an important impact on A/R_d (Heskel *et al.*, 2014), and thus presumably on tundra CUE. Similarly, in evergreen oak (*Quercus ilex*) trees experiencing water restriction under Mediterranean climatic conditions, reduction in R_d may explain up to 15% of net leaf carbon gain in summer (Sperlich *et al.*, 2016). Within tree canopies, there is also considerable variation in leaf R_d and R_d/A between upper (sun) and lower (shade) leaves, partly mirrored by R_n (Weerasinghe *et al.*, 2014). In other words, the adjustment of leaf day respiration with environmental or physiological conditions seems to be a significant actor of ecosystem CUE.

However, it should be recognized that there is a lack of quantitative experimental data on both R_d and CUE. Recently, isotopic labelling has been used to measure day respiration at the stand scale (mesocosm) and estimate CUE in sunflower (*Helianthus annuus*), and it has been shown that total mesocosm R_d was of the same order of magnitude as R_n (Gong *et al.*, 2017); furthermore, the data presented in this study suggest that leaf day respiration represents a significant proportion of mesocosm R_d, of *c.* 50–60%. Also, growth at high CO_2 led to a considerable increase in R_d (more than in R_n) causing a 8% decline in mesocosm CUE (Gong *et al.*, 2017).

Taken as a whole, day respiratory CO_2 efflux is a significant component of plant carbon budget, so that not taking into account the inhibition of respiration by light (i.e. the difference between day and night respiration) or changes in R_d with environmental conditions can lead to a substantial misestimation of plant or ecosystem CUE.

VIII. Conclusions and perspectives

The basic principles of the metabolic reorchestration taking place in the illuminated leaf are known, so that we now understand why the CO_2 efflux by respiration is lower in the light than in the dark (Fig. 1). Nevertheless, day respiratory metabolism appears to be rather complicated, because its regulation is dictated by interactions with photosynthesis, photorespiration and other pathways such as N assimilation. We do not know how these interactions are integrated by cellular metabolism and affect the flux of decarboxylation reactions. As a result, we do not know how to predict respiratory leaf CO_2 efflux in the light, and how it varies with environmental conditions. As a matter of fact, the lack of models that predict R_d (or R_n) is a persisting conundrum for studies of leaf gas exchange and plant carbon balance (for a specific discussion, see Atkin *et al.*, 2017). From a practical perspective, day respiration is also problematic because there is no convenient and accurate method that can be easily implementable in the field (Box 1). On the one hand, the Kok method, fast and not requiring CO_2 manipulation, can be confounded by other effects increasing leaf quantum yield at low light. On the other hand, the Laisk method can be compromised by changes in internal conductance and the response of day respiration to light and CO_2 mole fraction. Thus, there are some concerns regarding the validity of these two widely used techniques to obtain a precise value of R_d. In this review, it has been made apparent that day respiration has a nonnegligible impact on plant (and ecosystem) CUE, isotopic exchange, or calculations of internal conductance. Therefore, there is a need for innovative techniques and measurements to determine proper leaf day respiration rates. More generally, because of its central role in carbon and nitrogen balance, day respiration should now be viewed as a research topic of prime importance, and significant advances are to be expected in the near future.

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