Photosynthetic Oxygen Production: New Method Brings to Light Forgotten Flux

Oxygen (O_2) is evolved during photosynthetic electron transport when water is split by the oxygen-evolving complex to provide protons and electrons to the chloroplastic electron chain, thereby generating ATP and NADPH—the energy source and reducing power for plant metabolism. The majority of this chemical energy is used to drive photosynthetic carbon metabolism, which consists of ribulose-1,5-bisphosphate carboxylation (photosynthetic carbon reduction cycle) and oxygenation (photosynthetic carbon oxidation cycle); with a combined electron requirement = J_A. Four electrons are required for every O_2 evolved so that gross O_2 production (GOP) is related to linear electron transport (J) according to $J/4$. When linear electron transport is used only to drive CO_2 fixation, the consumption of O_2 and the release of CO_2 by photosynthetic carbon oxidation and mitochondrial respiration is such that net O_2 production (NOP) is equal to net CO_2 assimilation (A_{net}; provided the respiratory quotient is 1, but see Tcherkez et al., 2017).

Additionally, electrons can be used for alternative noncyclic electron transport (ANCET), including, for example, the photoreduction of O_2 itself forming reactive oxygen species (Mehler-peroxidase reactions or "water-water cycle"; Asada, 1999), chloroplastic anabolism (e.g. lipids; Stumpf et al., 1963), the reduction of oxaloacetate to malate (which is exported to the mitochondria; Scheibe, 2004), and nitrogen assimilation (Bloom et al., 1989). ANCET has been hypothesized both as a way to regulate ATP/NADPH ratio to meet the changing energy demands of cellular metabolism and as a mechanism to prevent photodamage through utilizing excess reductant when the photon flux density exceeds the energy requirement of CO_2 fixation (e.g. under high irradiance, cold temperatures, water stress closing stomata; e.g. Badger, 1985; Ort and Baker, 2002; Robinson, 1988). Importantly, there is no formal evidence for how electron flows interact, particularly under fluctuating light conditions (Morales et al., 2018).

As ANCET allows for greater rates of linear electron transport to be sustained, total electron transport (J_t) will be greater than J_A. Conversely, the effect on O_2 uptake will be dependent on the metabolic pathway involved. For example, in the Mehler-peroxidase reactions, there is no net change in O_2 so that NOP will remain equal to A_{net}. But in the reduction of nitrate, the ratio between N-linked O_2 production and O_2 consumption is highly dependent on the amino acid synthesized (Noctor and Foyer, 1998). In this case, NOP will not always equal A_{net} because O_2 and CO_2 may not be balanced in metabolism (Skillman, 2008). Consequently, concomitant measurements of CO_2 and O_2 fluxes are important to the understanding of how plants regulate the use of light energy, with different fates having very different metabolic outcomes.

The earliest measurements of O_2 evolution were unable to distinguish GOP from uptake of O_2 (Hill, 1937). The mass spectrometry method established by Mehler and Brown (1952) solved this problem by employing O_2 isotope tracers to independently monitor fluxes of $^{16}O_2$ and $^{18}O_2$. In this method, pure $^{18}O_2$ was supplied to the gas headspace of a closed chamber, and the decline in $^{18}O_2$ was attributed to O_2 uptake. O_2 evolved carries the same isotopic composition as the water from which it is generated; in this case, the dominant isotope in the water was ^{16}O (Fig. 1). The ^{18}O-labeling approach was further applied to leaf disks (e.g. Tourneux and Peltier, 1995), whole excised leaves (e.g. Volk and Jackson, 1972), and entire plants (Gerbaud and André, 1980), illuminating the fate of O_2 in vivo.

The limitation of closed gas exchange systems is that measurements can only be undertaken for short periods of time (seconds to minutes) before the CO_2 concentration is depleted. Consequently, CO_2:O_2 is not constant, which changes the relative rates of carboxylation and oxygenation so that estimates of GOP and O_2 uptake will be inaccurate. This limitation was overcome in the mass spectrometry approach by replacing CO_2 consumed through periodic influx of CO_2 into the chamber, allowing for steady-state quantification and extending the ability to measure O_2 fluxes under a range of conditions and physiological states (Canvin et al., 1980). At the same time, advances were being made in the use of chlorophyll fluorescence, which provides information on PSII quantum yield (Baker, 2008). Genty et al. (1989) provided the empirical link between fluorescence and electron transport rate, replacing the need to directly measure O_2 evolution. Chlorophyll fluorescence is now one of the most popular techniques in plant physiology because of its ease of use and relatively low cost. This has been aided by the capacity to multiplex fluorescence measurements with H_2O and CO_2 gas exchange in portable, commercially available instruments, opening up the possibility of measuring plant function outside of the laboratory. Consequently, in vivo measurements of O_2 fluxes have substantially declined over the last 20 years.

In this issue of *Plant Physiology*, Gauthier et al. (2018) remind us why it is so important to return our attention to O_2, providing us with a new, elegant open-path system to measure O_2 fluxes. Their method is a "reverse" isotopic approach, involving ^{18}O-labeling of leaf water rather than the air so that the isotopic composition of O_2 that is evolved during water splitting has a signature

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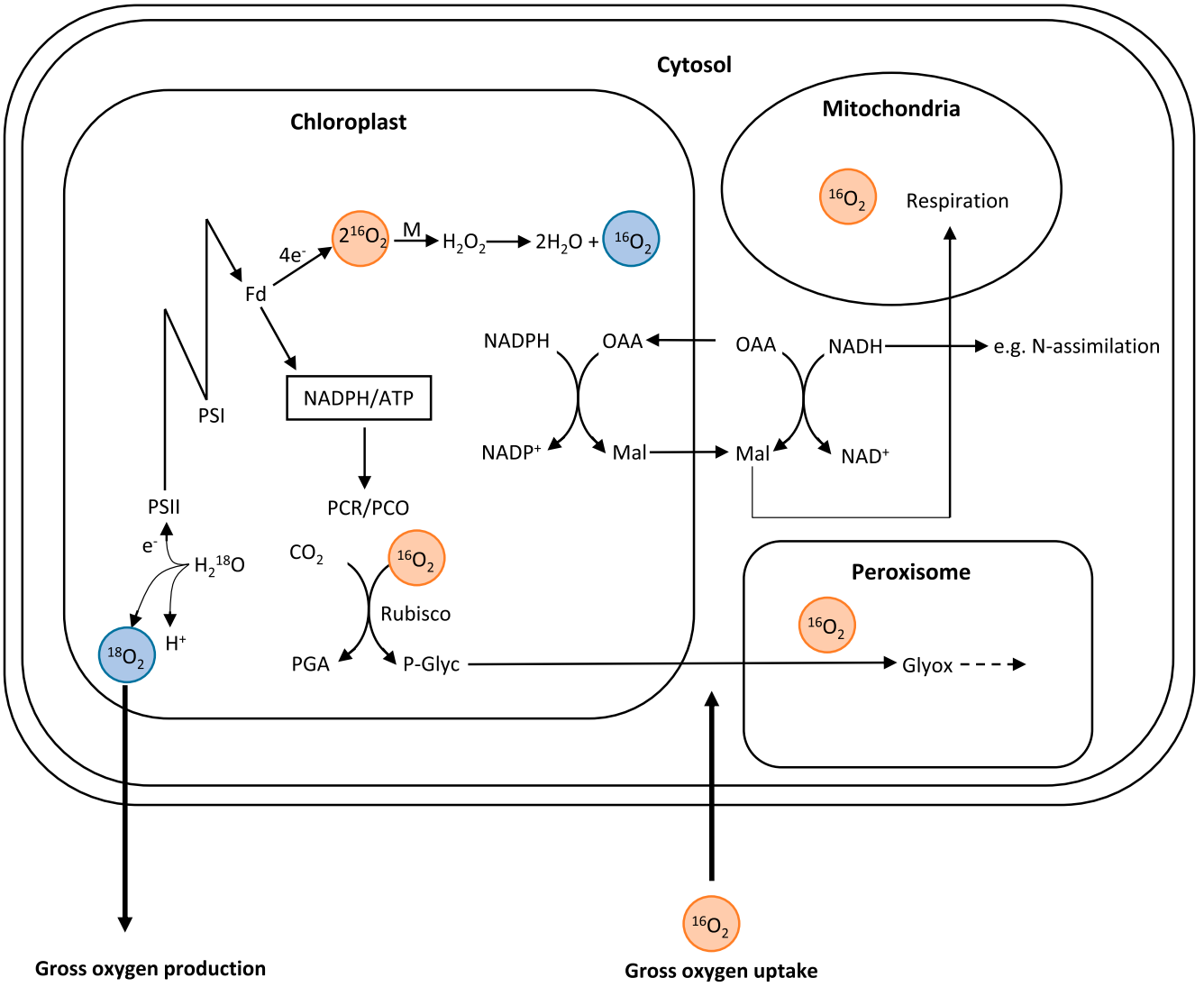

Figure 1. Simple representation of the reactions that can be involved in gross O_2 production and uptake of a photosynthesizing cell, showing how labeled ^{18}O water results in the production of $^{18}O_2$ in the approach developed by Gauthier et al. (2018). In the case of reactions within the peroxisome and mitochondria, this only represents net O_2 consumption, i.e. there is both uptake and release occurring. PSII, Photosystem II; PSI, Photosystem I; Fd, Ferredoxin; M, Mehler reaction; PCR; photosynthetic carbon reduction; PCO, photosynthetic carbon oxidation; PGA, 3-phosphoglycerate; P-Glyc, phosphoglycolate; Glyox, glyoxylate; OAA, oxaloacetate; Mal, malate.

very different to that of ambient O_2 (Fig. 1). The use of considerable ^{18}O enrichment is imperative since the contribution of NOP in a background of 21% O_2 is likely to be in the order of 0.05% (e.g. 100 μmol mol^{-1} NOP/210,000 μmol mol^{-1} ambient O_2), making it difficult ordinarily to accurately detect a change in $\delta^{18}O$ of O_2 associated with NOP in the air surrounding the leaf.

The method remains highly technical, requiring the use of three high-precision instruments. The isotopic composition and concentration of CO_2 and H_2O vapor are measured by laser spectroscopy, and the $\delta^{18}O_2$ and $\delta O_2/N_2$ (to estimate O_2 concentration) by mass spectrometry. A custom-made chamber is also required to house the excised leaf and its ^{18}O-labeled water source, which helps to prevent leaks across the gaskets from around the petiole. Importantly, the open gas exchange system improves the ability to achieve steady-state measurements, and labeling water versus the use of pure $^{18}O_2$ gas solves the affordability issue, which has greatly limited the adoption of open systems.

While chlorophyll fluorescence has become the popular option for measuring electron transport rate, it is not without assumptions. For example, it is frequently assumed that leaves absorb 84% of incident photons and that 50% of these photons are absorbed by PSII; however, this may not always be the case (Baker, 2008). This may lead to an overestimate of electron transport rate when computed from fluorescence compared

with measurements of GOP. Furthermore, accurate determination of J_A is particularly relevant for the estimation of mesophyll conductance, which was one application highlighted by Gauthier et al. (2018). The Mehler-peroxidase reactions, which have been shown to range from 0% to 30% (Driever and Baker, 2011), would lead to an overestimate of electron fluxes associated with the photosynthetic carbon reduction/oxygenation cycles in both methods. However, the advantage of the isotope labeling approach is that the contribution of the Mehler reaction to gross O_2 production can be quantified by coupling measurements of GOP with NOP (e.g. Furbank et al., 1982; see Fig. 1). Now that we have a renewed ability to measure O_2 fluxes, these assumptions should not be ignored.

Besides understanding the trade-off between efficiency and photoprotection for improved agricultural production (Murchie and Niyogi, 2011), the different electron fates have important implications for understanding global O_2 fluxes. Notably, O_2 uptake associated with photorespiration, mitochondrial respiration, and the Mehler-peroxidase reactions have different isotope fractionation factors (Guy et al., 1993) so that the quantification of individual pathway fluxes is needed to constrain estimates of global primary production from $\delta^{18}O$ information (Welp et al., 2011).

It is high time we revisited the measurement of O_2 fluxes, and the new method developed by Gauthier et al. (2018) provides us with the necessary capacity to do so.

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