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Modelling metabolic CO_2 evolution – a fresh perspective on respiration

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ABSTRACT

Respiration is a major contributor to net exchange of CO_2 between plants and the atmosphere and thus an important aspect of the vegetation component of global climate change models. However, a mechanistic model of respiration is lacking, and so here we explore the potential for flux balance analysis (FBA) to predict cellular CO_2 evolution rates. Metabolic flux analysis reveals that respiration is not always the dominant source of CO_2, and that metabolic processes such as the oxidative pentose phosphate pathway (OPPP) and lipid synthesis can be quantitatively important. Moreover, there is considerable variation in the metabolic origin of evolved CO_2 between tissues, species and conditions. Comparison of FBA-predicted CO_2 evolution profiles with those determined from flux measurements reveals that FBA is able to predict the metabolic origin of evolved CO_2 in different tissues/species and under different conditions. However, FBA is poor at predicting flux through certain metabolic processes such as the OPPP and we identify the way in which maintenance costs are accounted for as a major area of improvement for future FBA studies. We conclude that FBA, in its standard form, can be used to predict CO_2 evolution in a range of plant tissues and in response to environment.

Key-words: climate change; flux balance analysis; metabolic flux analysis; plant.

INTRODUCTION

The balance between photosynthesis and respiration is a key determinant of the carbon economy of plants and their exchange of CO_2 with the atmosphere (Reich 2010). As these carbon exchange fluxes are much greater than anthropogenic carbon release into the atmosphere (Canadell *et al*. 2007), quantitative measures of photosynthesis and plant respiration are a prerequisite for global climate change models. The response of these processes to increased temperature and decreased water availability also needs to be taken into account, and these effects will be critical in predicting the future performance of agricultural systems. While photosynthetic and respiratory CO_2 exchanges are amenable to experimental quantification at tissue level, empirical measures of CO_2 exchange cannot be reliably extrapolated to future environmental conditions. This leads to a requirement for models that can predict the impact of environmental conditions on the CO_2 fluxes in the metabolic network.

There is a marked disparity between the current state of modelling of photosynthesis and respiration. Photosynthesis modelling centres on the landmark biochemical model of Farquhar, von Caemmerer & Berry (1980, 2001). This mechanistic model consists of rate equations for the main biochemical processes of photosynthesis (Rubisco kinetics and electron transport rate accounting for light, temperature and photorespiration) and is capable of predicting net rates of photosynthetic CO_2 fixation in response to variables such as atmospheric CO_2 concentration, light intensity and temperature. The model predictions have been extensively validated and derivatives of the model are currently used in global climate models (Sitch *et al*. 2008; Smith & Dukes 2013).

In contrast, a mechanistic model of respiratory responses is currently lacking (Smith & Dukes 2013). Instead, respiration is usually predicted on the basis of its correlation with other processes. For example, in global climate change models, respiration is assumed to be proportional to leaf nitrogen content (Cox 2001; Reich *et al*. 2008). Other models correlate respiration with Rubisco content (de Pury & Farquhar 1997) or as a constant fraction of gross carbon gain (Waring, Landsberg & Williams 1998). These phenomenological measures of respiration lack predictive power, and unlike a mechanistic model, they do not generate new knowledge or testable hypotheses.

The other major approach in respiration modelling is the 'growth-respiration/maintenance-respiration paradigm' (Amthor 2000). While it may be useful to separate the respiratory provision of energy/reductant/carbon skeletons for growth and cell maintenance, quantification of growth and maintenance respiration is problematic and the paradigm lacks a mechanistic basis. Moreover, there are conceptual problems with separating growth and maintenance in this way: for example, the metabolic cost of cell maintenance, defined as the cost of maintaining the steady-state levels of metabolites and macromolecules and the electrochemical gradients across membranes, inevitably depends on the growth rate of immature tissues. There are also no distinct metabolic pathways for maintenance and growth. The idea of maintenance respiration as a distinct portion of respiration has long been debated and remains controversial (Thornley 2011).

CO_2 FLUXES AND RESPIRATION

The critical importance of being able to make reliable, mechanistic predictions about the effect of environment on

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respiratory rates and the necessity of integrating respiratory models into larger-scale ecosystem models was the focus of the 24th New Phytologist Symposium: 'Plant respiration and climate change: scaling from mitochondria to the globe', which was held in Oxford, UK, in 2010. The symposium highlighted several challenges that must be addressed in order to attain the goal of a mechanistic respiration model applicable across scales. But perhaps the starkest indicator of the nature of the challenge was that there was no general consensus among the participants of the meeting as to what the word 'respiration' actually means (Atkin, Millar & Turnbull 2010). This uncertainty arises partly because of the different scales at which the process can be considered and partly because of the multi-faceted nature of oxidative metabolism in plants (Plaxton & Podesta 2006).

For example, at the physiological scale, respiration is simply defined as the release of CO_2 that is distinct from photorespiratory CO_2, and it is not necessary to consider in detail where the CO_2 comes from. In contrast, at the biochemical level, the focus tends to be on oxidative metabolism. Respiration might then be defined in terms of oxygen consumption, which focuses on the processes that depend on oxygen as a terminal electron acceptor, or in terms of CO_2 production by the tricarboxylic acid cycle (TCA) cycle plus mitochondrial pyruvate dehydrogenase. Often the oxygen consumption and CO_2 production parameters are combined into a 'respiratory quotient', variations in which are often seen as evidence of switches of respiratory substrate, although there are many other explanations. As none of these definitions capture all of the light-independent metabolic processes that lead to the net production of CO_2, it becomes apparent that the focus needs to shift from the poorly defined term 'respiration' to 'net CO_2 evolution', where 'net CO_2 evolution' is defined as the sum of all the CO_2-producing steps minus the sum of all the CO_2-consuming steps, excluding photosynthesis and photorespiration.

Focusing on net CO_2 evolution leads to a network or holistic view of CO_2 production that corresponds to physiological reality. It also leads to the identification of two specific challenges for the modelling process. Firstly, it is necessary to identify all the metabolic processes that contribute to net CO_2 production and this is generally recognized to be extremely difficult (Barbour & Hanson 2009). Inspection of genome-scale models of Arabidopsis metabolism (Poolman *et al*. 2009; de Oliveira Dal'Molin *et al*. 2010a) reveals that there are in excess of 200 reactions that involve CO_2 or bicarbonate as a reactant, although the majority of these are not predicted to carry significant flux. Secondly, any predictive model needs to allow for differences between tissue types and the impact of a change in conditions on the processes that contribute to net CO_2 evolution. For example, net CO_2 evolution by the TCA cycle is considerably lower in leaves in the light than in heterotrophic tissues (Sweetlove *et al*. 2010); CO_2 release by 6-phosphogluconate dehydrogenase increases when exogenous nitrite stimulates the oxidative pentose phosphate pathway (OPPP; Averill, Bailey-Serres & Kruger 1998); and in growing tissues, the synthesis of different biomass components involves differential loss of CO_2 leading to substantial differences in carbon conversion efficiency (Chen & Shachar-Hill 2012). The recent progress that has been made in addressing these challenges is discussed below.

QUANTIFICATION OF BIOCHEMICAL PROCESSES LEADING TO CO_2 EVOLUTION BY METABOLIC FLUX ANALYSIS (MFA)

In order to understand the extent to which different processes contribute to net CO_2 evolution, it is necessary to quantify their rates in living tissues. The CO_2 fluxes associated with multiple steps in the network of central carbon metabolism can be deduced from stable isotope labelling experiments using MFA (Kruger, Masakapalli & Ratcliffe 2012; O'Grady *et al*. 2012). There are two experimental approaches: steady-state MFA, in which the system is analysed when it has reached a metabolic and isotopic steady state, and non-steady-state analysis, in which the fluxes are deduced from a labelling time-course (Ratcliffe & Shachar-Hill 2006). Tracing the dynamics of label incorporation is experimentally and computationally more demanding than steady-state analysis as it requires a time-course of both metabolite concentrations and labelling patterns. However, the approach is essential for the analysis of photoautotrophic systems because steady-state labelling with $^{13}CO_2$ yields an uninformative labelling pattern (Roscher, Kruger & Ratcliffe 2000; Shastri & Morgan 2007). In fact, achieving an isotopic and metabolic steady state in leaves is likely to be difficult because of the slow turnover of metabolite pools (Lattanzi *et al*. 2012; Szecowka *et al*. 2013) and the complications introduced by the light-dark cycle, so even if a precursor other than the usual substrate is used, it would be difficult to satisfy the conditions for steady-state MFA (Tcherkez *et al*. 2012). Accordingly, despite the complexity of the analysis, dynamic labelling methods are essential for photoautotrophic networks, and the first flux maps of light-driven carbon fixation have now been published (Young *et al*. 2011; Szecowka *et al*. 2013). In contrast, steady-state MFA is a well-established technique that has been applied to a wide range of heterotrophic and mixotrophic plant tissues typically using positionally labelled glucose as a substrate.

The metabolic networks that are used in MFA to explain the observed labelling of metabolic intermediates and end-products include several steps that either assimilate or release CO_2 (Fig. 1). The relative contribution of these processes to the net production or assimilation of CO_2 is shown in Fig. 2a for a range of tissues, and two general conclusions can be drawn from the data. Firstly, there are considerable differences between different tissues and different species. Clearly, Rubisco activity dominates the CO_2 balance in photoautotrophic *Synechocystis*, but it also makes a major contribution to the carbon balance in the embryos of Arabidopsis, soybean and *Brassica napus*. However, among these embryos, soybean shows a markedly different pattern of CO_2 release, reflecting the higher protein content and lower oil content of the embryo. Similarly, different tissues of maize show different patterns of CO_2 release, while heterotrophic

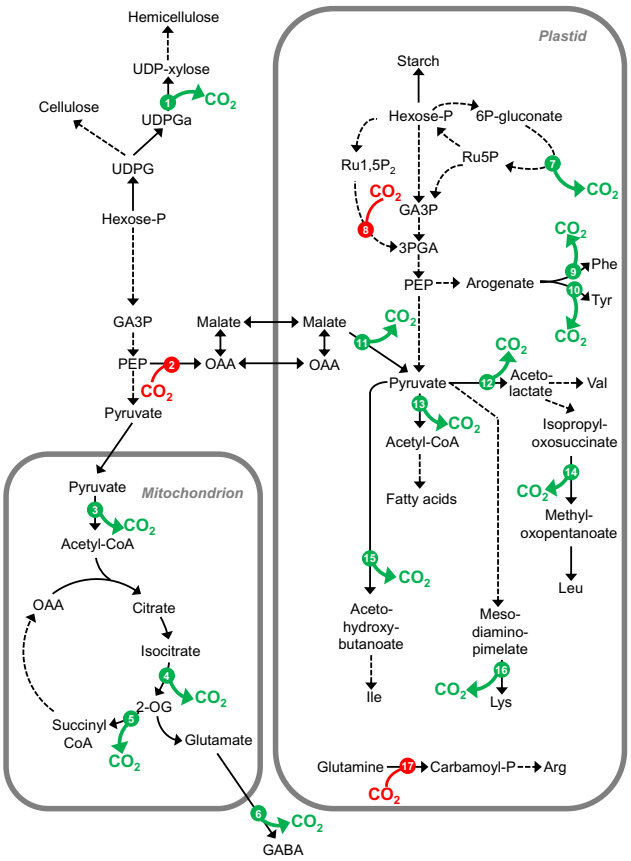

Figure 1. The central metabolic network of a growing heterotrophic/mixotrophic plant cell illustrating some of the major reactions in which CO_2 is produced or consumed. Numbered green circles indicated CO_2-producing reactions and numbered red circles indicated CO_2-consuming reactions. Solid lines indicate a single reaction and dashed lines indicate multiple reactions. Reactions catalysed by the following enzymes are illustrated: 1, UDP-glucoronate decarboxylase; 2 PEP carboxylase; 3, pyruvate dehydrogenase (mitochondrial); 4, isocitrate dehydrogenase; 5, 2-oxoglutarate dehydrogenase; 6, glutamate decarboxylase; 7, phosphogluconate dehydrogenase; 8, Rubisco; 9, arogenate dehydratase; 10, arogenate dehydrogenase; 11, NADP-dependent malic enzyme; 12, acetolactate synthase; 13, pyruvate dehydrogenase (plastidial); 14, spontaneous reaction; 15, acetolactate synthase; 16, diaminopimelate decarboxylase; 17, carbamoyl-phosphate synthase. Abbreviations: 2-OG, 2-oxoglutarate; 3PGA, 3-phosphoglycerate; GA3P, glyceraldehyde 3-phosphate; GABA, γ-aminobutyric acid; OAA, oxaloacetate; PEP, phospho*enol*pyruvate; Ru, ribulose; UDPG, UDP-glucose; UDPGa, UDP-glucuronate.

cell cultures show a distinctive pattern in which CO_2 release is dominated by the TCA cycle and the OPPP. The second general conclusion is that the TCA cycle, which is taken here to include the CO_2 output from mitochondrial pyruvate dehydrogenase as well as the outputs from isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase, does not necessarily dominate CO_2 production in many tissues. Thus, the picture that emerges from these MFA studies is one in which the contribution of different processes to the CO_2 balance is highly variable.

MFA has also been used to a limited extent to compare different genotypes within a species (Arabidopsis embryos, maize root tips) and to investigate the impact of changes in growth conditions on CO_2 consumption and production in soybean cotyledons, Arabidopsis cell suspensions and *Chlorella* (Fig. 2b). Significant changes were observed in some of these studies. For example, the increased importance of the TCA cycle as a source of CO_2 in the embryos of the Arabidopsis *wri1-1* mutant and in Arabidopsis cells at elevated temperature. In other studies, however, a change in conditions, for example, the imposition of nitrogen limitation on *Chlorella* cells, had very little effect on the proportion of CO_2 produced by different processes. The limited number of studies in the literature reflects the time-consuming nature of MFA, but at this point, it appears that there may be greater variability in the proportion of CO_2 produced and consumed between species and tissues, than between growth conditions. Such a conclusion, which can only be provisional at this stage, would reflect the expected biosynthetic specialization of different tissues and species, and the need for a robust central metabolic network in the face of suboptimal growth conditions.

Although the implementation of fully quantitative MFA presents technical challenges, and especially so in photoautrophic tissues, the multiplicity of CO_2-consuming and CO_2-producing steps makes it difficult to assess their contribution reliably from less rigorous assessments of labelling data. For example, ratios of $^{14}CO_2$ released from positionally labelled glucose and gluconate provide a relatively rapid indication of the relative fluxes through the pathways of carbohydrate oxidation in plants (Harrison & Kruger 2008). However, extracting reliable estimates of the intracellular CO_2 fluxes from such data would require a comprehensive flux analysis equivalent in its complexity to conventional MFA. This is certainly possible as it has been shown that CO_2 release data from analogous experiments with ^{13}C-labelled substrates in microorganisms can be used as the basis for complete MFA when combined with biomass (Yang, Wittmann & Heinzle 2006a,b), but it does not provide a shortcut to an assessment of the relative importance of the various contributions to net CO_2 production.

In an alternative approach (Barbour & Hanson 2009; Tcherkez *et al.* 2012), pulse-labelling with positionally ^{13}C-labelled substrates and/or measurements of the carbon isotopic ratio ($\delta^{13}C$) of CO_2 and can be used to identify the major contributions to net CO_2 evolution in roots and leaves (Bathellier *et al.* 2009; Gessler *et al.* 2009; Priault, Wegener & Werner 2009). The quantitative interpretation of these experiments is not trivial, and for heterotrophic tissues, it is arguable that steady-state MFA is the better method for extracting comprehensive and reliable flux data from a labelling experiment. However, for photoautotrophic tissues, where labelling time-courses provide the only route to intracellular fluxes and where the application of MFA is particularly challenging, the case for the alternative approach is stronger (Tcherkez *et al.* 2012). In a substantial study of illuminated leaves, positionally ^{13}C-labelled substrates and deuterium isotope effects were used to reveal non-cyclic fluxes in

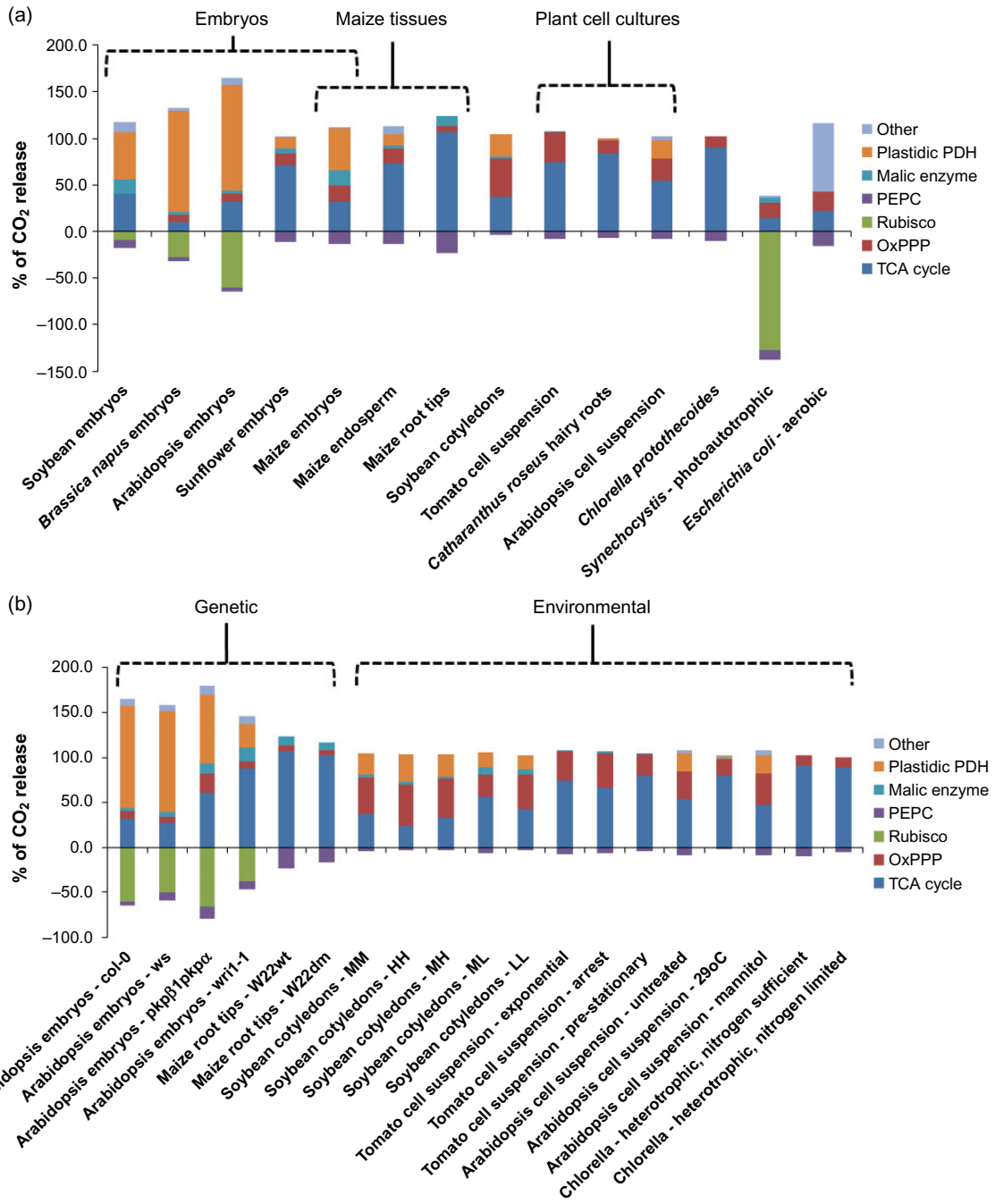

Figure 2. Metabolic flux analysis (MFA) measurements of CO_2 fluxes in plant tissues. Contributions to the net evolution of CO_2 are expressed as a percentage of net CO_2 production, and the sources and sinks for CO_2 are grouped into seven categories: TCA cycle (isocitrate dehydrogenase, 2-oxoglutarate dehydrogenase plus mitochondrial pyruvate dehydrogenase), OxPPP (6-phosphogluconate dehydrogenase), Rubisco, PEPC (phospho*enol*pyruvate carboxylase), malic enzyme (plastidic, cytosolic and mitochondrial isoforms), pastidic PDH (plastidic pyruvate dehydrogenase) and other (pentan synthesis, amino acid biosynthesis, fermentation). (a) Data for soybean embryos (Allen, Ohlrogge & Shachar-Hill 2009), *Brassica napus* embryos (Schwender *et al.* 2006), Arabidopsis embryos (Lonien & Schwender 2009), sunflower embryos (Alonso *et al.* 2007a), maize embryos (Alonso, Dale & Shachar-Hill 2010), maize endosperm (Alonso, Val & Shachar-Hill 2011) maize root tips (Alonso *et al.* 2007b), soybean cotyledons (Sriram *et al.* 2004), tomato cell suspensions (Rontein *et al.* 2002), *Catharanthus roseus* hairy root cultures (Sriram, Fulton & Shanks 2007), Arabidopsis cell suspensions (Williams *et al.* 2010), heterotrophic cultures of *Chlorella protothecoides* (*Xiong et al. 2010)*, photoautotrophic cultures of *Synechocystis* (Young *et al.* 2011) and aerobic cultures of *Escherichia coli* (Chen *et al.* 2011). (b) CO_2 flux data allowing a comparison of genotypes or showing the effect of a change in conditions for Arabidopsis embryos (Lonien & Schwender 2009), maize root tips (Alonso *et al.* 2007b), soybean cotyledons (Sriram *et al.* 2007; Iyer *et al.* 2008), tomato cell suspensions (Rontein *et al.* 2002), Arabidopsis cell suspensions (Williams *et al.* 2010) and heterotrophic cultures of *C. protothecoides* (Xiong *et al.* 2010). Note that certain MFA studies were excluded from this analysis, either because the published data did not provide sufficient information or because the studies were closely related to those already selected.

the TCA cycle (Tcherkez *et al*. 2009). Quantification of fluxes was attempted in terms of probability or transmission coefficients, but the underlying usefulness of these terms for deducing fluxes is questionable as they are not easily related to conventional fluxes. Thus, although the qualitative interpretation of the measurements is informative, it seems that this approach will eventually be supplanted by the comprehensive analysis of labelling time-courses that has been pioneered in *Synechocystis* (Young *et al*. 2011) and recently extended to Arabidopsis leaves (Szecowka *et al*. 2013).

THE CASE FOR FLUX BALANCE MODELLING AS A PREDICTOR OF NET CO_2 EVOLUTION

The available analyses of fluxes in the central metabolic network of plants reveal the extent to which the metabolic origin of released CO_2 varies between different tissues and under different conditions. Thus, any model of the effect of environment on net CO_2 evolution at the plant level needs to be able to predict the response of each of the different CO_2-evolving processes within the tissues that make up the plant. In green tissues, this will naturally require integration with models that predict the change in net photosynthetic CO_2 assimilation rate.

The large size of the metabolic network that must be considered in accounting for net CO_2 evolution more or less precludes the construction of a fully mechanistic enzyme kinetic model given the impracticality of quantifying the kinetic parameters of a large number of enzymes (Schallau & Junker 2010; Rohwer 2012). Indeed, it is a commonly held viewpoint that plant respiratory regulation is too complex for a mechanistic representation (Gifford 2003). This may be true at the level of enzyme kinetics, but there are other modelling approaches that may be able to provide appropriately fine-grained prediction of metabolic fluxes without kinetic parameters. The most promising alternative is currently flux balance analysis (FBA), which provides a framework for flux analysis based on reaction stoichiometry. By making a steady-state assumption and applying constraints on the inputs and outputs to the metabolic system, it is possible to predict the internal flux states of all reactions in the network by applying an optimization criterion (Orth, Thiele & Palsson 2010; Sweetlove & Ratcliffe 2011). Typically, the constraints are the requirement to synthesize biomass of appropriate proportions and at a certain rate, and the optimization criterion is usually based on metabolic efficiency, either minimization of enzyme machinery costs (and hence minimal total reaction fluxes in the network) or maximum carbon conversion efficiency. This approach can thus predict fluxes of each reaction in the network that consume or produce CO_2. Moreover, if there is information on how the constraints respond to environmental changes, for example, the effect of temperature or water stress on biomass composition, then FBA can make predictions about the relationship between environment and net CO_2 evolution rates. Note that the success of FBA is dependent on a complete and correct list of enzymatic reactions and the accuracy of the experimentally measured constraints.

The idea of using a stoichiometrically balanced metabolic network model to account for net CO_2 evolution during the synthesis of new biomass and cell maintenance was first proposed in the 1970s (Penning de Vries, Brunstin & Vanlaar 1974). More recently, the approach was taken up and extended in a model that considered CO_2 evolution in C_3 leaves in the light and the dark (Buckley & Adams 2011). However, in both studies, highly simplified metabolic network models were used in which the overall CO_2 stoichiometry of lumped sets of reactions representing biochemical processes (photosynthesis, photorespiration, oxidative phosphorylation, etc.) was considered. While this is perfectly acceptable from a modelling perspective, the simplification can inadvertently reduce the co-dependence of biochemical processes if all the appropriate branch points between them are not considered. Additionally, the metabolic network representation is not compatible with that used currently in the FBA field, in which every individual metabolic reaction is accounted for. The majority of recent FBA models are 'genome-scale' and thus include all the enzymatic steps that are encoded by the genome of the organism (de Oliveira Dal'molin & Nielsen 2013; Seaver, Henry & Hanson 2012). Given the accelerating appearance of such large-scale FBA models, the question we wish to address in this review is whether these models can be used to predict net CO_2 evolution in plants.

DOES FLUX BALANCE ANALYSIS PREDICT REALISTIC DISTRIBUTIONS OF NET CO_2 EVOLUTION?

Although the number of FBA studies of large-scale plant metabolic networks is increasing rapidly, there are relatively few which consider tissue types or conditions for which there is also equivalent MFA data. Two studies explicitly compare MFA and FBA flux predictions for the same cell/tissue: an Arabidopsis cell suspension culture (Williams *et al*. 2010) and cultured *B. napus* embryos (Hay & Schwender 2011).

In both cases, the FBA models were constrained to an experimentally determined carbon conversion efficiency value, and hence the amount of CO_2 evolved in absolute terms is a set parameter, not a prediction of the model. Although the composition of biomass was similarly constrained by experimental data, the routes taken to synthesize that biomass and the contribution of different biochemical processes to the overall CO_2 evolution budget were not fixed in the FBA model and can be viewed as model predictions. The comparative data are shown in Fig. 3. It can be seen that FBA successfully predicts some aspects of the net CO_2 evolution budget determined by MFA, but not others.

For example, FBA is able to predict that respiration (TCA cycle plus mitochondrial pyruvate dehydrogenase) dominates the CO_2 evolution profile of heterotrophic Arabidopsis cell suspension cultures (Fig. 3a). The FBA model is also able to predict the dramatic decrease in the contribution of respiratory CO_2 release to the total in *B. napus* embryos (Fig. 3b). However, in both cases, there are quantitative differences between the FBA predictions and those estimated by MFA.

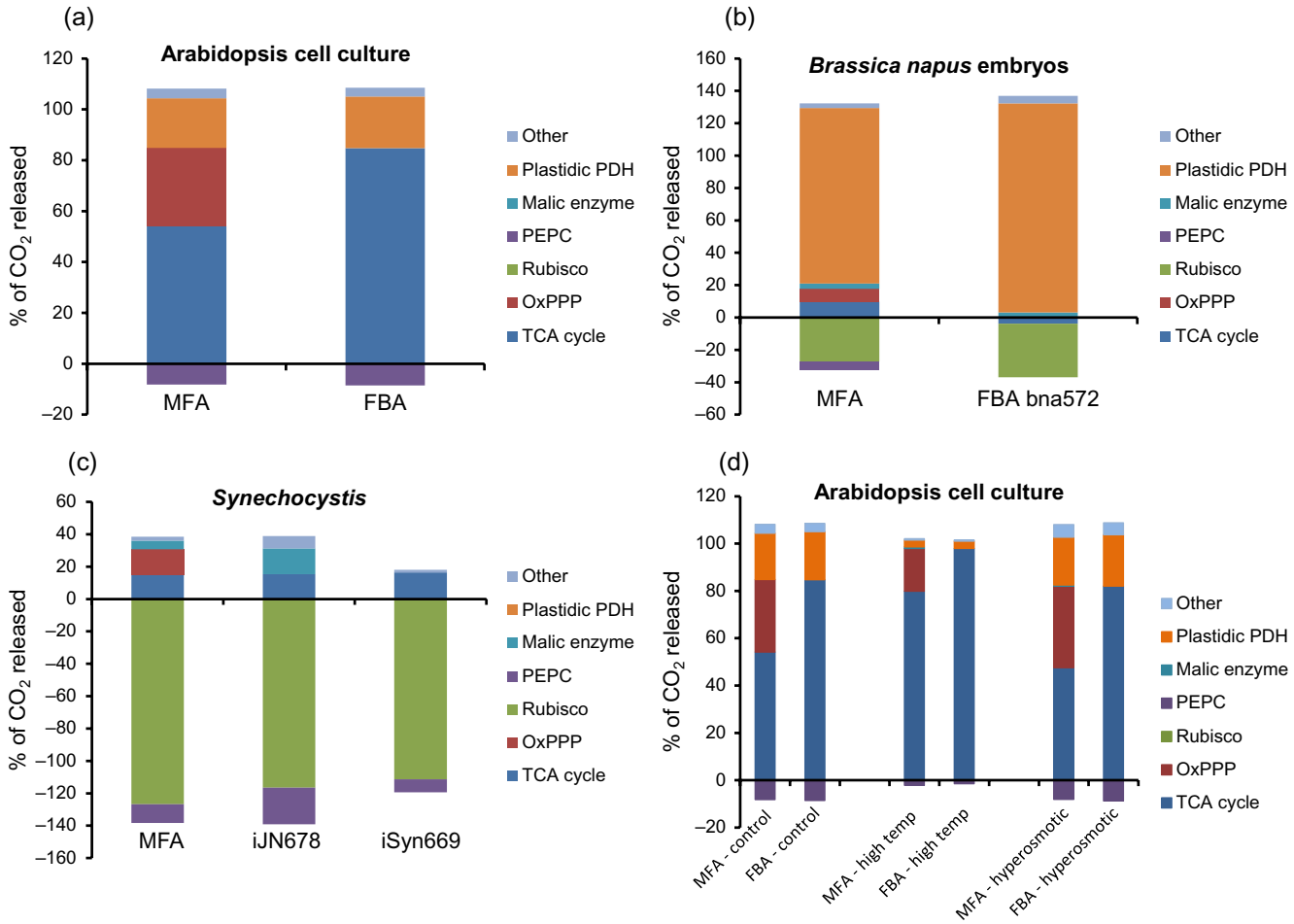

Figure 3. Comparison of the CO_2 evolution profile predicted by flux balance analysis (FBA) with that calculated from metabolic flux analysis (MFA)-derived flux maps. (a) Heterotrophic Arabidopsis cell suspension culture under standard growth conditions. Data from Williams *et al.*, 2010. (b) *Brassica napus* embryos supplied with organic nitrogen. MFA data from Schwender *et al.* (2006). FBA data from Hay & Schwender (2011). (c) Photoautotrophic *Synechocystis* sp. PCC6803. MFA data from Young *et al.* (2011). FBA data from Nogales *et al.* (2012) (iJN678) and Montagud *et al.* (2010) (iSyn669). (d) Heterotrophic Arabidopsis cell suspension culture cultured under standard (control) conditions, under high temperature (29 °C) and under hyperosmotic stress; data from Williams *et al.* (2010). In order to facilitate comparison between FBA and MFA, the simulations reported in this paper were repeated using identical biomass constraints.

In *B. napus* embryos, a negative net respiratory flux is predicted by FBA in contrast to the small net positive flux estimated by MFA. This is because the embryo culture medium contains organic nitrogen sources (Gln, Glu, Ala, Asn) mimicking the supply of organic nitrogen from the mother plant, and the high amino acid availability allows glutamate to feed into a reverse-acting TCA cycle (Schwender, Shachar-Hill & Ohlrogge 2006). In the FBA prediction, the reverse flux to citrate is greater in relation to the forward fluxes through pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase, resulting in a net negative CO_2 yield from the TCA 'cycle' (Hay & Schwender 2011).

The other substantial differences in the CO_2 evolution profile between *B. napus* embryos and Arabidopsis are broadly captured by FBA. The greater contribution of plastidial pyruvate dehydrogenase to CO_2 evolution in *B. napus*, reflecting the greater amount of fatty acid being synthesized, is accurately predicted, as is the relatively smaller contribution of other biosynthetic processes to CO_2 evolution (Fig. 3a,b). The contribution of Rubisco in the photoheterotrophic embryo is also well predicted.

Similarly, FBA broadly captures the CO_2 evolution distribution in the heterotrophic Arabidopsis cell culture, with the exception of one substantial inaccuracy: the OPPP (Fig 3a). FBA predicts that the oxidative branch of the OPPP carries zero flux, whereas this pathway accounts for 31% of the total CO_2 release in the MFA solution. As a consequence, flux through the TCA cycle is substantially overestimated in the FBA flux prediction. In fact, flux through the OPPP is absent in the vast majority of the plant FBA models and we will discuss the implications of this in subsequent sections.

It is also important to assess whether FBA can accurately predict the distribution of CO_2 release among different biochemical processes in photosynthetic tissues. At the time of writing, there are no published large-scale experimental flux maps for a higher plant leaf with which to compare

published FBA studies of both C3 and C4 photosynthesis, although the experimental and mathematical framework for such flux calculations have recently been demonstrated (Szecowka *et al*. 2013). There is an excellent instationary flux analysis of the photosynthetic cyanobacterium *Synechocystis* sp. PCC6803 (Young *et al*. 2011), which can be used as a point of reference for two FBA models: iSyn669 (Montagud *et al*. 2010) and iJN678 (Nogales *et al*. 2012).

Again, broadly speaking, FBA is able to replicate the relative contribution of different processes to overall CO_2 release with some inaccuracies and differences (Fig. 3c). Some of these discrepancies may be related to the fact that these are independent studies and different experimental constraints were applied to the models. However, other differences, such as the CO_2 flux attributed to biosynthesis in the FBA solutions, reflect the use of different routes for the biosynthesis of certain amino acids. Both FBA models provide an accurate assessment of the 15% contribution of mitochondrial CO_2 production to total CO_2 evolution, but neither model captures the 16% of the total CO_2 exchange that originates from the OPPP. As a result, the iSyn699 substantially underestimates the CO_2 released from photoautotrophic *Synechocystis* metabolism. In contrast, the iJN678 model overestimates CO_2 release, partly due to the high CO_2 release by biosynthetic processes and partly due to an unexpectedly high flux through malic enzyme. It is not clear why malic enzyme carries such a high flux, although it should be noted that the value for this flux is highly variable in different solutions to the model (the flux range is 0–0.164 mmol g DW^{-1} h^{-1}). A high flux variability in different solutions of the same model indicates that the model is insufficiently constrained to provide a precise value for this particular flux.

In addition to predicting changes in the fluxes through CO_2-evolving processes in different cell/tissue types, FBA must also be able to predict the effect of altered environment. Thus far, this has only been examined in Arabidopsis cell suspension cultures where it was reported that FBA can accurately predict the change in flux distribution in central metabolism caused by high temperature and hyperosmotic stress treatments (Williams *et al*. 2010). Consequently, FBA provides a reasonably accurate prediction of changes in the CO_2 evolution profile under these conditions (Fig 3d), successfully predicting the increase in mitochondrial respiration under high temperature conditions and the changes in CO_2 evolution due to plastidic pyruvate dehydrogenase activity under both conditions. However, the failure to predict a flux in the OPPP skews the CO_2 evolution profile, especially under hyperosmotic conditions where MFA shows that its contribution to overall CO_2 evolution increases (Fig. 3d).

IMPROVING THE PREDICTIVE ACCURACY OF FBA: THE MAINTENANCE ENERGY PROBLEM

The predictive accuracy of the CO_2 fluxes in FBA models has yet to be fully established, but the evidence to date is encouraging. The approach clearly has potential as a tool to predict plant net CO_2 evolution and this information can be extracted from FBA studies in their standard form. However, while FBA can readily predict changes in the CO_2 evolution profile that are driven by the synthesis of biomass of different composition, for example, the difference between an oil-rich developing embryo and a photosynthetic bacterium, the ability of FBA to predict the effects of environment on the CO_2 evolution profile has only been tested on an Arabidopsis cell suspension culture. Nevertheless, the cell suspension comparison does reveal that FBA can, in principle, accurately predict the effects of altered environment on the CO_2 evolution profile. Moreover, it is apparent that the effects of environment on the relative contribution of different processes to net CO_2 evolution is relatively minor, at least in heterotrophic Arabidopsis cell suspension cultures, in comparison to the differences observed between tissues types and species.

Although FBA is remarkably accurate in some predictions, it is not able to reliably predict the quantity of CO_2 released from all biochemical processes and it is clear that improvements are necessary before it can be used as part of global plant CO_2 exchange models. Perhaps, the biggest deficiency is the consistent failure of FBA models to predict a flux in the oxidative branch of the OPPP. This is significant because in some tissues, such as soybean cotyledons and tomato cell suspensions, and under some conditions, such as hyperosmotic stress, the OPPP is a major contributor to overall CO_2 release (Fig. 2). The oxidative branch of the OPPP is one of the major sources of cellular reductant in the form of NADPH. The fact that FBA models can synthesize biomass in sufficient quantities without a flux through the oxidative branch of the OPPP illustrates that other dehydrogenase enzymes (e.g. isocitrate dehydrogenase, malate dehydrogenase, etc.) can satisfy the NADPH demand and are chosen above the OPPP by the optimization algorithm. Structural refinement of the models may alter this conclusion, but the situation could also change when NADPH demands additional to biomass synthesis are included, such as antioxidant activity (Foyer & Noctor 2011) and lipid turnover/resynthesis.

Such processes come under the category of cell maintenance. Although some, but by no means all, FBA models include a maintenance cost as a constraint, this is usually based on reported values for cell maintenance that are derived from experimental relationships between respiration and growth rates. Recently, a more interesting way of accounting for maintenance has emerged based on a requirement to match the carbon consumed by the model with the experimental value (Poolman *et al*. 2009; Hay & Schwender 2011). The assumption is that any shortfall in carbon consumed once biomass synthesis is accounted for must be due to respiratory oxidation of carbohydrate to meet the ATP cost of cell maintenance. In modelling terms, this equates to the inclusion of an additional, generic ATPase reaction in the model and allowing the flux through this reaction to vary until the carbon consumption gap is closed.

However, whichever method is used for the estimation of maintenance cost, it is equated to a cost expressed solely in terms of ATP. This leads to the assumption that CO_2

evolution due to cell maintenance has its origin in the TCA cycle (Amthor 2000). The prediction of zero OPPP flux in FBA models calls this assumption into question and suggests that maintenance energy costs must be divided between ATP and NADPH. If this is not performed, TCA cycle CO_2 evolution is overestimated and CO_2 evolution from the OPPP is underestimated, as is the case in current FBA model predictions.

Correct accounting for maintenance costs thus emerges as a major area for improvement of existing FBA models. Simply constraining maintenance costs to experimentally determined maintenance CO_2 evolution rates is not particularly useful for several reasons. Firstly, it provides no predictive power as to how this cost will vary under different conditions. Secondly, experimental determination of maintenance costs in plant tissues is beset with difficulties (Amthor 2000). Thirdly, measurements of the cost of maintenance generally provide no information about the partitioning between ATP and NADPH demand. Ideally, then, maintenance costs would be more directly captured in the flux balance model.

In FBA terms, maintenance essentially means anything that is not directly involved in the net synthesis of biomass. This therefore includes the cost of resynthesis of macromolecules that turn over, with protein and mRNA turnover likely to be quantitatively the most important. Additionally, maintenance includes the cost of energizing the plasma membrane, and endomembranes such as the tonoplast, to permit active transport of metabolites and macromolecules. Some of these transport costs can be directly linked to the requirements of biomass synthesis provided the model is adequately compartmented and appropriate transport mechanisms are included. However, the inherent proton leak of all membranes, which is likely to be highly dependent on environmental conditions, and factors such as metabolite cycling across membranes need to be accounted for as part of the maintenance calculation. Another potentially significant maintenance cost is that of metabolite cleansing – the removal of unwanted, toxic side products of enzymatic reactions and random chemical events (Seaver *et al*. 2012). Finally, as already mentioned, the reductant cost of antioxidant activity must also be considered.

It will be a substantial challenge to incorporate all of these processes into FBA models. There are essentially two approaches. One option is to estimate their rates experimentally, which requires substantial effort (Piques *et al*. 2009), and for some processes, such as flux through the ascorbate-glutathione cycle, there has been very little in the way of quantitative analysis. Alternatively, it may be possible to model the processes and these models can be integrated into the larger FBA model. For example, because proton leak across membranes is a consequence of the biophysics of the lipid bilayer, it should be possible to predict the effect of altered environment on the leak rate using a biophysical model. Nevertheless, is clear that some processes will remain extremely difficult to predict. Protein turnover, for example, is very much a feature of the specific protein and its local environment, and there are order of magnitude differences in the observed rates of turnover of different proteins. Hence, it is not realistic to expect a generalizable rule that relates the global rate of protein turnover, which is the relevant constraint for FBA modelling, to environment. In such cases, experimentally measured information will be required to constrain the model appropriately.

PERSPECTIVES

In assessing the merits of FBA as a tool to predict net CO_2 evolution rates of plant tissues, we have been restricted by the number of tissue types and environmental conditions for which experimentally constrained metabolic flux maps are available as a point of comparison/validation. This is mainly because of the limited number of experimental systems that are suitable for the more tractable steady-state stable isotope MFA approach. In particular, because flux quantification in photosynthetic tissues requires the more challenging analysis of labelling time-courses, there is a shortage of quantitative flux maps for such tissues. So, although there have been several detailed FBA studies of photosynthetic tissues of higher plants (Montagud *et al*. 2010; de Oliveira Dal'Molin *et al*. 2010b; Chang *et al*. 2011; Saha, Suthers & Maranas 2011; Nogales *et al*. 2012), at the time of writing, the only comparable experimental flux map of a sufficiently large-scale metabolic network is for the cyanobacterium *Synechocystis* (Young *et al*. 2011). Although the requirement for calculation of fluxes from dynamic labelling patterns is both experimentally and computationally more demanding, there are several groups that have been developing the necessary experimental and analytical tools for flux analysis in leaves in the light (Huege *et al*. 2007; Hasunuma *et al*. 2010; Keerberg *et al*. 2011; Lattanzi *et al*. 2012) and it is likely that flux maps will emerge in due course. Recently, a major step forward towards this goal was made with the publication of an analysis of the dynamic label redistribution of label from $^{13}CO_2$ supplied to Arabidopsis rosette leaves, from which a small set of fluxes were calculated (Szecowka *et al*. 2013). This paper establishes the experimental, analytical and mathematical frameworks that will allow a more systematic analysis of metabolic network fluxes in leaves and will facilitate the assessment of FBA for predicting CO_2 evolution profiles in the dominant tissues of higher plants.

In summary, it is clear that FBA has the potential to predict fluxes through the CO_2-consuming and CO_2-generating processes in plant tissues, and based on existing work, it should be capable of predicting how the CO_2 evolution profile will change in response to environment. Given the increasing interest in FBA as a tool to examine plant metabolic networks and the acceleration of sequencing of diverse plant genomes, there is every reason to expect that a more sophisticated, species-specific prediction of plant net CO_2 evolution could ultimately be incorporated into higher-level ecosystem models.

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REFERENCES

Allen D.K., Ohlrogge J.B. & Shachar-Hill Y. (2009) The role of light in soybean seed filling metabolism. *The Plant Journal* **58,** 220–234.

Alonso A.P., Goffman F.D., Ohlrogge J.B. & Shachar-Hill Y. (2007a) Carbon conversion efficiency and central metabolic fluxes in developing sunflower (*Helianthus annuus L.*) embryos. *The Plant Journal* **52,** 296–308.

Alonso A.P., Raymond P., Hernould M., Rondeau-Mouro C., de Graaf A., Chourey P., Lahaye M., Shachar-Hill Y., Rolin D. & Dieuaide-Noubhani M. (2007b) A metabolic flux analysis to study the role of sucrose synthase in the regulation of the carbon partitioning in central metabolism in maize root tips. *Metabolic Engineering* **9,** 419–432.

Alonso A.P., Dale V.L. & Shachar-Hill Y. (2010) Understanding fatty acid synthesis in developing maize embryos using metabolic flux analysis. *Metabolic Engineering* **12,** 488–497.

Alonso A.P., Val D.L. & Shachar-Hill Y. (2011) Central metabolic fluxes in the endosperm of developing maize seeds and their implications for metabolic engineering. *Metabolic Engineering* **13,** 96–107.

Amthor J.S. (2000) The McCree-de Wit-Penning de Vries-Thornley respiration paradigms: 30 years later. *Annals of Botany* **86,** 1–20.

Atkin O., Millar H. & Turnbull M. (2010) Plant respiration in a changing world. *New Phytologist* **187,** 269–272.

Averill R.H., Bailey-Serres J. & Kruger N.J. (1998) Co-operation between cytosolic and plastidic oxidative pentose phosphate pathways revealed by 6-phosphogluconate dehydrogenase-deficient genotypes of maize. *The Plant Journal* **14,** 449–457.

Barbour M.M. & Hanson D.T. (2009) Stable carbon isotopes reveal dynamics of respiratory metabolism. *New Phytologist* **181,** 243–245.

Bathellier C., Tcherkez G., Bligny R., Gout E., Cornic G. & Ghashghaie J. (2009) Metabolic origin of the delta^{13}C of respired CO_2 in roots of *Phaseolus vulgaris*. *New Phytologist* **181,** 387–399.

Buckley T.N. & Adams M.A. (2011) An analytical model of non-photorespiratory CO_2 release in the light and dark in leaves of C_3 species based on stoichiometric flux balance. *Plant, Cell & Environment* **34,** 89–112.

Canadell J.G., Le Quere C., Raupach M.R., Field C.B., Buitenhuis E.T., Ciais P., Conway T.J., Gillett N.P., Houghton R.A. & Marland G. (2007) Contributions to accelerating atmospheric CO_2 growth from economic activity, carbon intensity, and efficiency of natural sinks. *Proceedings of the National Academy of Sciences of the United States of America* **104,** 18866–18870.

Chang R.L., Ghamsari L., Manichaikul A., *et al.* (2011) Metabolic network reconstruction of Chlamydomonas offers insight into light-driven algal metabolism. *Molecular Systems Biology* **7,** 518.

Chen X. & Shachar-Hill Y. (2012) Insights into metabolic efficiency from flux analysis. *Journal of Experimental Botany* **63,** 2343–2351.

Chen X., Alonso A.P., Allen D.K., Reed J.L. & Shachar-Hill Y. (2011) Synergy between ^{13}C-metabolic flux analysis and flux balance analysis for understanding metabolic adaptation to anaerobiosis in *E. coli*. *Metabolic Engineering* **13,** 38–48.

Cox P. (2001) Description of the 'TRIFFID' dynamic global vegetation model. *Hadley Centre Technical Note* **24,** 1–16.

Farquhar G.D., von Caemmerer S. & Berry J.A. (1980) A biochemical model of photosynthetic CO_2 assimilation in leaves of C_3 species. *Planta* **149,** 78–90.

Farquhar G.D., von Caemmerer S. & Berry J.A. (2001) Models of photosynthesis. *Plant Physiology* **125,** 42–45.

Foyer C.H. & Noctor G. (2011) Ascorbate and glutathione: the heart of the redox hub. *Plant Physiology* **155,** 2–18.

Gessler A., Tcherkez G., Karyanto O., Keitel C., Ferrio J.P., Ghashghaie J., Kreuzwieser J. & Farquhar G.D. (2009) On the metabolic origin of the carbon isotope composition of CO_2 evolved from darkened light-acclimated leaves in *Ricinus communis*. *New Phytologist* **181,** 374–386.

Gifford R.M. (2003) Plant respiration in productivity models: conceptualisation, representation and issues for global terrestrial carbon-cycle research. *Functional Plant Biology* **30,** 171–186.

Harrison P.W. & Kruger N.J. (2008) Validation of the design of feeding experiments involving [^{14}C]substrates used to monitor metabolic flux in higher plants. *Phytochemistry* **69,** 2920–2927.

Hasunuma T., Harada K., Miyazawa S., Kondo A., Fukusaki E. & Miyake C. (2010) Metabolic turnover analysis by a combination of *in vivo* ^{13}C-labelling from $^{13}CO_2$ and metabolic profiling with CE-MS/MS reveals rate-limiting steps of the C3 photosynthetic pathway in *Nicotiana tabacum* leaves. *Journal of Experimental Botany* **61,** 1041–1051.

Hay J. & Schwender J. (2011) Computational analysis of storage synthesis in developing *Brassica napus* L. (oilseed rape) embryos: flux variability analysis in relation to ^{13}C metabolic flux analysis. *The Plant Journal* **67,** 513–525.

Huege J., Sulpice R., Gibon Y., Lisec J., Koehl K. & Kopka J. (2007) GC-EI-TOF-MS analysis of *in vivo* carbon-partitioning into soluble metabolite pools of higher plants by monitoring isotope dilution after $^{13}CO_2$ labelling. *Phytochemistry* **68,** 2258–2272.

Iyer V.V., Sriram G., Fulton D.B., Zhou R., Westgate M.E. & Shanks J.V. (2008) Metabolic flux maps comparing the effect of temperature on protein and oil biosynthesis in developing soybean cotyledons. *Plant, Cell & Environment* **31,** 506–517.

Keerberg O., Ivanova H., Keerberg H., Parnik T., Talts P. & Gardestrom P. (2011) Quantitative analysis of photosynthetic carbon metabolism in protoplasts and intact leaves of barley. Determination of carbon fluxes and pool sizes of metabolites in different cellular compartments. *Bio Systems* **103,** 291–301.

Kruger N.J., Masakapalli S.K. & Ratcliffe R.G. (2012) Strategies for investigating the plant metabolic network with steady-state metabolic flux analysis: lessons from an Arabidopsis cell culture and other systems. *Journal of Experimental Botany* **63,** 2309–2323.

Lattanzi F.A., Ostler U., Wild M., Morvan-Bertrand A., Decau M.L., Lehmeier C.A., Meuriot F., Prud'homme M.P., Schaufele R. & Schnyder H. (2012) Fluxes in central carbohydrate metabolism of source leaves in a fructan-storing C3 grass: rapid turnover and futile cycling of sucrose in continuous light under contrasted nitrogen nutrition status. *Journal of Experimental Botany* **63,** 2363–2375.

Lonien J. & Schwender J. (2009) Analysis of metabolic flux phenotypes for two Arabidopsis mutants with severe impairment in seed storage lipid synthesis. *Plant Physiology* **151,** 1617–1634.

Montagud A., Navarro E., Fernandez de Cordoba P., Urchueguia J.F. & Patil K.R. (2010) Reconstruction and analysis of genome-scale metabolic model of a photosynthetic bacterium. *BMC Systems Biology* **4,** 156.

Nogales J., Gudmundsson S., Knight E.M., Palsson B.O. & Thiele I. (2012) Detailing the optimality of photosynthesis in cyanobacteria through systems biology analysis. *Proceedings of the National Academy of Sciences of the United States of America* **109,** 2678–2683.

O'Grady J., Schwender J., Shachar-Hill Y. & Morgan J.A. (2012) Metabolic cartography: experimental quantification of metabolic fluxes from isotopic labelling studies. *Journal of Experimental Botany* **63,** 2293–2308.

de Oliveira Dal'molin C.G. & Nielsen L.K. (2013) Plant genome-scale metabolic reconstruction and modelling. *Current Opinion in Biotechnology* **24,** 271–277.

de Oliveira Dal'Molin C.G., Quek L.E., Palfreyman R.W., Brumbley S.M. & Nielsen L.K. (2010a) AraGEM, a genome-scale reconstruction of the primary metabolic network in Arabidopsis. *Plant Physiology* **152,** 579–589.

de Oliveira Dal'Molin C.G., Quek L.E., Palfreyman R.W., Brumbley S.M. & Nielsen L.K. (2010b) C4GEM, a genome-scale metabolic model to study C4 plant metabolism. *Plant Physiology* **154,** 1871–1885.

Orth J.D., Thiele I. & Palsson B.O. (2010) What is flux balance analysis? *Nature Biotechnology* **28,** 245–248.

Penning de Vries F., Brunstin A.H. & Vanlaar H.H. (1974) Products, requirements and efficiency of biosynthesis – a quantitative approach. *Journal of Theoretical Biology* **45,** 339–377.

Piques M., Schulze W.X., Hohne M., Usadel B., Gibon Y., Rohwer J. & Stitt M. (2009) Ribosome and transcript copy numbers, polysome occupancy and enzyme dynamics in Arabidopsis. *Molecular Systems Biology* **5,** 314.

Plaxton W.C. & Podesta F.E. (2006) The functional organization and control of plant respiration. *Critical Reviews in Plant Science* **25,** 1–40.

Poolman M.G., Miguet L., Sweetlove L.J. & Fell D.A. (2009) A genome-scale metabolic model of Arabidopsis and some of its properties. *Plant Physiology* **151,** 1570–1581.

Priault P., Wegener F. & Werner C. (2009) Pronounced differences in diurnal variation of carbon isotope composition of leaf respired CO_2 among functional groups. *New Phytologist* **181,** 400–412.

de Pury D.G.G. & Farquhar G.D. (1997) Simple scaling of photosynthesis from leaves to canopies with the errors of big-leaf models. *Plant, Cell & Environment* **20,** 537–557.

Ratcliffe R.G. & Shachar-Hill Y. (2006) Measuring multiple fluxes through plant metabolic networks. *The Plant Journal* **45,** 490–511.

Reich P.B. (2010) Climate change. The carbon dioxide exchange. *Science* **329,** 774–775.

Reich P.B., Tjoelker M.G., Pregitzer K.S., Wright I.J., Oleksyn J. & Machado J.L. (2008) Scaling of respiration to nitrogen in leaves, stems and roots of higher land plants. *Ecology Letters* **11,** 793–801.

Rohwer J.M. (2012) Kinetic modelling of plant metabolic pathways. *Journal of Experimental Botany* **63,** 2275–2292.

Rontein D., Dieuaide-Noubhani M., Dufourc E.J., Raymond P. & Rolin D. (2002) The metabolic architecture of plant cells. Stability of central metabolism and flexibility of anabolic pathways during the growth cycle of tomato cells. *Journal of Biological Chemistry* **277,** 43948–43960.

Roscher A., Kruger N.J. & Ratcliffe R.G. (2000) Strategies for metabolic flux analysis in plants using isotope labelling. *Journal of Biotechnology* **77,** 81–102.

Saha R., Suthers P. & Maranas C. (2011) *Zea mays* i RS1563: a comprehensive genome-scale metabolic reconstruction of maize metabolism. *PLoS ONE* **6,** e21784.

Schallau K. & Junker B.H. (2010) Simulating plant metabolic pathways with enzyme-kinetic models. *Plant Physiology* **152,** 1763–1771.

Schwender J., Shachar-Hill Y. & Ohlrogge J.B. (2006) Mitochondrial metabolism in developing embryos of *Brassica napus*. *Journal of Biological Chemistry* **281,** 34040–34047.

Seaver S.M., Henry C.S. & Hanson A.D. (2012) Frontiers in metabolic reconstruction and modeling of plant genomes. *Journal of Experimental Botany* **63,** 2247–2258.

Shastri A.A. & Morgan J.A. (2007) A transient isotopic labeling methodology for ^{13}C metabolic flux analysis of photoautotrophic microorganisms. *Phytochemistry* **68,** 2302–2312.

Sitch S., Huntingford C., Gedney N., *et al.* (2008) Evaluation of the terrestrial carbon cycle, future plant geography and climate-carbon cycle feedbacks using five Dynamic Global Vegetation Models (DGVMs). *Global Change Biology* **14,** 2015–2039.

Smith N.G. & Dukes J.S. (2013) Plant respiration and photosynthesis in global-scale models: incorporating acclimation to temperature and CO_2. *Gobal Change Biology* **19,** 45–63.

Sriram G., Fulton D.B., Iyer V.V., Peterson J.M., Zhou R., Westgate M.E., Spalding M.H. & Shanks J.V. (2004) Quantification of compartmented metabolic fluxes in developing soybean embryos by employing biosynthetically directed fractional ^{13}C labeling, two-dimensional [^{13}C, ^{1}H] nuclear magnetic resonance, and comprehensive isotopomer balancing. *Plant Physiology* **136,** 3043–3057.

Sriram G., Fulton D.B. & Shanks J.V. (2007) Flux quantification in central carbon metabolism of *Catharanthus roseus* hairy roots by ^{13}C labeling and comprehensive bondomer balancing. *Phytochemistry* **68,** 2243–2257.

Sweetlove L.J. & Ratcliffe R.G. (2011) Flux-balance modelling of plant metabolism. *Frontiers in Plant Science* **2,** 38.

Sweetlove L.J., Beard K.F., Nunes-Nesi A., Fernie A.R. & Ratcliffe R.G. (2010) Not just a circle: flux modes in the plant TCA cycle. *Trends in Plant Science* **15,** 462–470.

Szecowka M., Heise R., Tohge T., *et al.* (2013) Metabolic fluxes in an illuminated Arabidopsis rosette. *The Plant Cell* **25,** 694–714.

Tcherkez G., Mahe A., Gauthier P., Mauve C., Gout E., Bligny R., Cornic G. & Hodges M. (2009) *In folio* respiratory fluxomics revealed by ^{13}C isotopic labeling and H/D isotope effects highlight the noncyclic nature of the tricarboxylic acid 'cycle' in illuminated leaves. *Plant Physiology* **151,** 620–630.

Tcherkez G., Boex-Fontvieille E., Mahe A. & Hodges M. (2012) Respiratory carbon fluxes in leaves. *Current Opinion in Plant Biology* **15,** 308–314.

Thornley J.H. (2011) Plant growth and respiration re-visited: maintenance respiration defined – it is an emergent property of, not a separate process within, the system – and why the respiration : photosynthesis ratio is conservative. *Annals of Botany* **108,** 1365–1380.

Waring R.H., Landsberg J.J. & Williams M. (1998) Net primary production of forests: a constant fraction of gross primary production? *Tree Physiology* **18,** 129–134.

Williams T.C.R., Poolman M.G., Howden A.J., Schwarzlander M., Fell D.A., Ratcliffe R.G. & Sweetlove L.J. (2010) A genome-scale metabolic model accurately predicts fluxes in central carbon metabolism under stress conditions. *Plant Physiology* **154,** 311–323.

Xiong W., Liu L., Wu C., Yang C. & Wu Q. (2010) ^{13}C tracer and GC-MS analysis reveal metabolic flux distribution in oleaginous microalga *Chlorella protothecoides*. *Plant Physiology* **154,** 1001–1011.

Yang T.H., Wittmann C. & Heinzle E. (2006a) Respirometric ^{13}C flux analysis – part II: *in vivo* flux estimation of lysine-producing *Corynebacterium glutamicum*. *Metabolic Engineering* **8,** 432–446.

Yang T.H., Wittmann C. & Heinzle E. (2006b) Respirometric ^{13}C flux analysis, part I: design, construction and validation of a novel multiple reactor system using on-line membrane inlet mass spectrometry. *Metabolic Engineering* **8,** 417–431.

Young J.D., Shastri A.A., Stephanopoulos G. & Morgan J.A. (2011) Mapping photoautotrophic metabolism with isotopically nonstationary ^{13}C flux analysis. *Metabolic Engineering* **13,** 656–665.

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