

REVIEW PAPER

# Insights into metabolic efficiency from flux analysis

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## Abstract

The efficiency of carbon and energy flows throughout metabolism defines the potential for growth and reproductive success of plants. Understanding the basis for metabolic efficiency requires relevant definitions of efficiency as well as measurements of biochemical functions through metabolism. Here insights into the basis of efficiency provided by  $^{13}\text{C}$ -based metabolic flux analysis (MFA) as well as the uses and limitations of efficiency in predictive flux balance analysis (FBA) are highlighted.  $^{13}\text{C}$ -MFA studies have revealed unusual features of central metabolism in developing green seeds for the efficient use of light to conserve carbon and identified metabolic inefficiencies in plant metabolism due to dissipation of ATP by substrate cycling. Constraints-based FBA has used efficiency to guide the prediction of the growth and actual internal flux distribution of plant systems. Comparisons in a few cases have been made between flux maps measured by  $^{13}\text{C}$ -based MFA and those predicted by FBA assuming one or more maximal efficiency parameters. These studies suggest that developing plant seeds and photoautotrophic microorganisms may indeed have patterns of metabolic flux that maximize efficiency. MFA and FBA are synergistic toolsets for uncovering and explaining the metabolic basis of efficiencies and inefficiencies in plant systems.

**Key words:** Carbon conversion efficiency, efficiency, metabolic flux analysis, plant metabolism.

## Introduction

The flow of carbon and energy through metabolism is at the heart of plant life. From  $\text{CO}_2$  fixation to the elaboration and turnover of complex polymers and secondary products, metabolic fluxes interconvert hundreds of thousands of organic compounds (Fiehn, 2002). Biologically, the efficiency of these processes defines the potential for growth and reproductive success. Practically, efficiency determines agricultural and biotechnological yields; and ecologically it constrains food webs and the global carbon cycle. Accordingly, the efficiencies of whole cells and organisms have been discussed for over a century (Rubner, 1902). Most analyses of efficiency have historically been limited to estimates of overall material and energy input/output balances (Calow, 1977), and therefore interest in efficiency has almost been entirely in ecological, allometric, and applied agricultural and biotechnological studies of heterotrophic cells and organisms.

Efficiency has been examined for some detailed biochemical processes such as energy transduction in photosynthetic light utilization (Zhu *et al.*, 2010; Rosenthal *et al.*,

2011) or ATP generation in respiration (Gonzalez-Meler *et al.*, 2009; Macfarlane *et al.*, 2009; Millar *et al.*, 2011), and this has yielded insight into the origins of particular causes of energy and material dissipation. However, it is the functioning of entire metabolic networks that determines the efficiency of cells and organisms. Thus understanding metabolic efficiency requires methods for quantifying the detailed flows of energy and material through mid- to large-scale networks. Metabolic flux analysis (MFA) brings together the necessary theoretical and experimental tools for such analyses (Stephanopoulos *et al.*, 1998; Schwender, 2009).

Applications of MFA and related tools to plant metabolism, especially central metabolism, have in recent years begun to provide the necessary level of detail to analyse the biochemical basis of efficiency (Libourel and Shachar-Hill, 2008; Allen *et al.*, 2009a; Kruger and Ratcliffe, 2009; Bar-Even *et al.*, 2012; O'Grady *et al.*, 2012; Kruger *et al.*, 2012; Rohwar, 2012; Seaver *et al.*, 2012; Steuer *et al.*, 2012). In addition, computational tools for exploring the capabilities

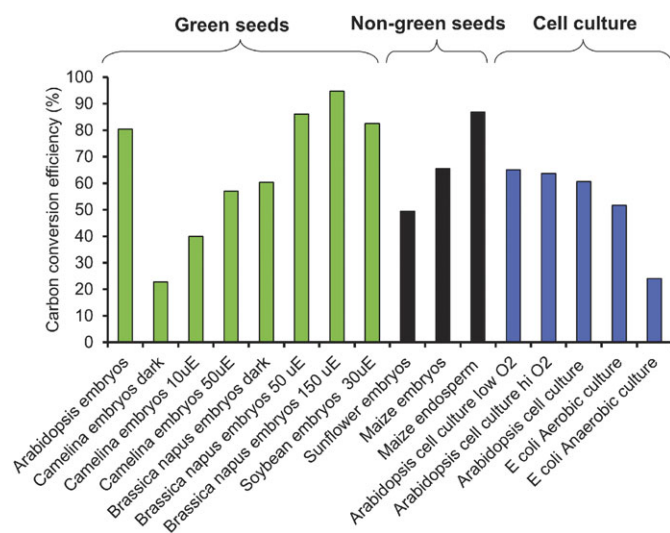
of large metabolic networks are now being applied to genome scale-plant metabolic networks (Poolman *et al.*, 2009; Hay and Schwender, 2011a, b). Such tools facilitate the detailed accounting needed to calculate and dissect accurately the potential and actual efficiencies. Indeed one important class of computational modelling of complete metabolic networks (constraints-based flux balance analysis; Sweetlove and Ratcliffe, 2011) usually assumes optimized efficiency to predict metabolic flux patterns.

In addition to computational tools, understanding the basis for metabolic efficiency requires relevant definitions of efficiency as well as measurements of biochemical function that are both detailed and integrated across the major fluxes through metabolism. Here different measures of efficiency in the metabolism of plant cells and tissues are considered and the insights that metabolic flux analysis can give into the basis for efficiency as well as the uses and limitations of efficiency in predictive flux analysis are highlighted.

## Carbon conversion efficiency

Carbon utilization provides a straightforward definition of metabolic efficiency as the ratio of useful products to substrates imported. Expressed as a percentage, carbon conversion efficiency (CCE) provides a practical basis for comparing the conversion of metabolic substrates into biomass and secreted products in growing cells and tissues (Calow, 1977). CCE highlights the proportion of resources devoted to accumulation of structural, storage, and reproductive biomass. In biotechnological settings, yield (product made per substrate used, which is closely analogous to CCE) is crucial to the practical success of microbial fermentations and is a central target for process optimization (Doran, 1995). In agricultural and ecological settings, the focus on yield has, with the exception of comparing incident with captured light energy (Blankenship *et al.*, 2011), mostly been on gross productivity rather than on CCE. This reflects the fact that for photosynthetic tissues the substrate (CO<sub>2</sub>) is not considered a cost, as well as the difficulties of measuring and analysing CCE in plants, which normally requires feeding plants with [<sup>14</sup>C]substrates and analysing <sup>14</sup>C labelling in plant biomass components.

However, when such detailed analyses have been possible, CCE has been used in heterotrophic and photoheterotrophic plant cells and tissues to compare metabolic strategies (Boyle and Morgan, 2011), to compare actual and potential productivity (Grafahrend-Belau *et al.*, 2009), and as a measure of the differences between developing seeds of different crop species. Figure 1 includes the CCE of product formation during development of the seeds of crop and model plant species from the literature, with the addition of unpublished data on *Camelina sativa*. CCE highlights the differences among seeds of similar and divergent composition. Thus the seeds of crops with high oil contents that are green during development, such as *Brassica napus* (oilseed rape) and soybean, have higher CCEs than sunflower whose seeds cannot use light. Within green oil-storing seeds,



**Fig. 1.** Carbon conversion efficiencies during seed filling in crop and model species. Taken from published studies (listed in Table 1) where developing seeds, embryos, or cells were cultured *in vitro* under conditions that mimic the substrate supply, light levels, growth rates, and composition of biomass made for seeds developing *in planta*. Ranges for *B. napus* and *C. sativa* are shown for embryos grown at different light levels; the middle of each range in these cases is closest to the growth and composition *in planta*. Experimental SD for the reproducibility of the measurements is normally <10%, although biomass composition analyses can also suffer from systematic errors. CCE typically has SDs within 5% of the estimated value.

*Camelina* stands out as having a lower CCE—indicating the potential for improvement of yield in this species, which is an oilseed crop plant that has not been bred for yield to anything like the same degree as other domesticated crops. Selection by breeding during human history has never been based explicitly on CCE, and early selection for domestication traits including but not limited to the size of individual seeds (Zohary and Hopf, 2000) may be largely independent of it. In modern times, selection for yield per plant, harvest index, and yield per hectare (Araus *et al.*, 2008) may be expected to favour varieties with higher seed CCE.

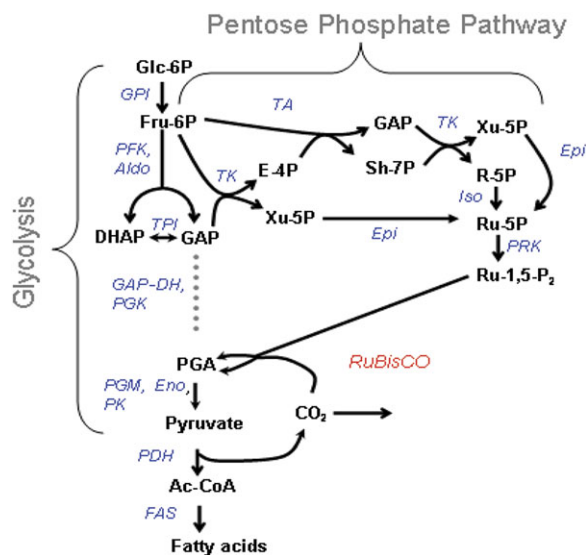
While relevant for considering natural and anthropogenic selection, gross CCE is a limited measure of metabolic efficiency because it treats all carbon substrates and products as identical. This limitation can be seen when comparing the efficiency of seeds that store mostly starch with those that store substantial amounts of oil and/or protein. Thus maize endosperm (and probably other starch-storing grains) has a much higher CCE than other non-green and even most light-utilizing seed tissues. This is more a reflection of the fact that converting sugar substrates into starch is energetically inexpensive and involves relatively few steps compared with making oil or protein from the same substrates. Thus the conversion of hexose into starch requires the equivalent of two ATP hydrolysis reactions per glucosyl residue (hexose → hexose-phosphate → UDPglucose) and is redox-neutral, whereas oil and protein synthesis and polymerization are relatively costly by comparison.

## Contribution of metabolic flux analysis to understanding efficiency

Thus a more detailed analysis of metabolism is needed to interpret measured CCEs. At a first level, the products made and substrates used must be known. However, to identify the reactions and pathways that lower CCE and obtain measurements of their contributions requires a quantitative mapping of central metabolic processes.  $^{13}\text{C}$ -based MFA measures the fluxes through central metabolism and therefore yields information for understanding CCE. MFA studies of developing *B. napus* seeds (Schwender *et al.*, 2003, 2004, 2006) revealed that their relatively high CCE is based on several unusual metabolic features: a negligible rate of  $\text{CO}_2$  emission from the mitochondrial oxidative decarboxylations of the tricarboxylic acid (TCA) cycle; little flux through the oxidative reactions of the pentose phosphate pathway; and a partial recapture, or rather recycling, of the  $\text{CO}_2$  emitted during fatty acid synthesis (at pyruvate dehydrogenase) through the operation of Rubisco but without significant flux through the complete Calvin–Benson–Bassham cycle. Associated with these is the use of light energy to supply reductant (the high energy electrons of NADPH) and usable free energy (in the currency of ATP). By meeting much of the demand for these co-substrates, light capture allows lower fluxes through the oxidative pentose phosphate (OPPP) pathway and TCA cycle, and increases CCE. MFA studies of *Arabidopsis* and soybean (Allen *et al.*, 2009b; Lonien and Schwender, 2009) have shown that, to different extents, the seeds of these green oilseed plants also have high CCE values by the same underlying mechanisms. The relatively low CCE values for *C. sativa* are associated with high TCA fluxes (L. Carey and Y. Shachar-Hill, unpublished results). In contrast, CCE is lower in oil-producing seeds that are not green, including sunflower and the embryos of maize (Alonso *et al.*, 2007a, 2010). Supporting the role of light in explaining CCE is the observation that *B. napus* embryos grown in the dark share a CCE close to 50% with the non-green oilseeds (Goffman *et al.*, 2005). Figure 2 shows the ‘Rubisco bypass’ that operates in green seeds exposed to low light levels and which was shown by Schwender *et al.* (2004) using  $^{13}\text{C}$ -MFA to approximately halve the  $\text{CO}_2$  emitted by developing *B. napus* embryos.

Another feature of metabolic efficiency highlighted by  $^{13}\text{C}$ -MFA studies in plants is the dissipation of ATP by substrate (futile) cycling. By consuming a significant proportion of catabolically produced ATP (e.g. Dieuaide-Noubhani *et al.*, 1995), futile cycling could lower the CCE. This means that if ATP dissipation were minimized, respiration and its associated  $\text{CO}_2$  evolution would probably be lower due to the lowering of total ATP demands. The contributions of MFA to understanding futile cycling and the uncertainties about its importance are reviewed elsewhere in this issue (O’Grady *et al.*, 2012).

Although flux thorough different pathways can be considered to influence efficiency, it is important to note that pathways do not operate in isolation, and both CCE



**Fig. 2.** The ‘Rubisco bypass’ for the metabolic transformation of sugars into fatty acids. In this pathway, conversion of hexose-P to pentose-P via the non-oxidative steps of the pentose phosphate pathway and the subsequent formation of PGA by Rubisco bypasses the glycolytic enzymes GAP-DH and PGK while recycling half of the  $\text{CO}_2$  released by PDH. PGA is then further metabolized to pyruvate, thence to Ac-CoA and into fatty acids. Metabolites: Ac-CoA, acetyl coenzyme-A; DHAP, dihydroxyacetone 3-phosphate; E-4P, erythrose 4-phosphate; Fru-6P, fructose 6-phosphate; GAP, glyceraldehyde 3-phosphate; Glc-6P, glucose 6-phosphate; PGA, 3-phosphoglyceric acid; R-5P, ribose 5-phosphate; Ru-1,5-P<sub>2</sub>, ribulose 1,5-bisphosphate; Ru-5P, ribulose 5-phosphate; Sh-7P, sedoheptulose 7-phosphate; Xu-5P, xylulose 5-phosphate. Enzymes: Aldo, fructose bisphosphate aldolase; Eno, 2-phosphoglycerate enolase; Epi, xylulose 5-phosphate epimerase; FAS, fatty acid synthase; PGM, phosphoglyceromutase; GAP-DH, 3-phosphoglycerate dehydrogenase; GPI, phosphoglucose isomerase; Iso, ribose 5-phosphate isomerase; PDH, pyruvate dehydrogenase; PFK, phosphofructokinase; PK, pyruvate kinase; PGK, phosphoglycerate kinase; PRK, phosphoribulokinase; TA, transaldolase; TK, transketolase; TPI, triose phosphate isomerase.

and energy conversion efficiency (ECE) are emergent properties of the whole flux map. For example, the glycolytic route from sugars to fatty acid has a nominal CCE of 66%, whereas the Rubisco bypass captures 80%; however, in the first case, enough reductant is produced for lipid synthesis whereas in the second light-derived electrons are needed. Likewise, the TCA and OPPP cycles both have nominal CCEs of 0%, but their operation must be considered in the context of the cellular demands for reductant and ATP.

## Energy conversion efficiency

The use of MFA to understand CCE shows that CCE is limited by the carbon stoichiometries of the biochemical mechanisms of central and biosynthetic metabolism as well as by requirements for cofactors. To go further in comparing



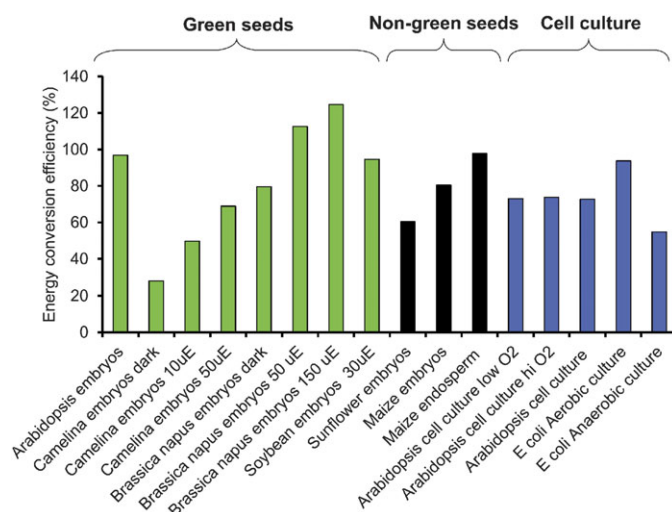
the metabolic efficiencies of cells and tissues, one must consider additional physical conservation laws, which relate to these cofactors or co-substrates [FADH<sub>2</sub> and NAD(P)H for redox and ATP and other NTPs for free energy]. The additional constraints are the conservation of energy and electrons, meaning that free energy and redox equivalents are conserved. Redox conservation constrains CCE when carbon compounds are the metabolic sources of reducing equivalents; thus, for example, making lipid (carbon oxidation state approximately -2) from carbohydrate (carbon oxidation state ~0) requires the release of oxidized products (in aerobic conditions this is CO<sub>2</sub>, oxidation state +4) so the maximal CCE for making fatty acid moieties from carbohydrates in heterotrophic cells is limited to close to 2/3. This constraint on CCE is lifted if non-carbon sources of reductant are available, usually the linear electron transport light reactions of photosynthesis as discussed above for green seeds.

In the case of making products that are more oxidized than the substrates taken up, such as cell cultures that produce organic acids, CCE may be lowered by the requirement to secrete reduced carbon byproducts (McKinlay *et al.*, 2007). When oxygen or other non-carbon sinks for significant quantities of reductant (e.g. nitrate) are available, this constraint is lifted. However, during hypoxia, if non-carbon sources and sinks for electrons/reductant are limiting, this imposes severe limits. Fermentation lowers CCEs (*Escherichia coli* under anaerobic conditions in Fig. 1) and this is avoided by a global down-regulation of metabolism when *Arabidopsis* cells are grown at lower oxygen levels.

Energy conservation is an obvious restriction on metabolism, and the second law of thermodynamics limits the conversion of free energy in biochemical as in all chemical and physical transformations. Thermodynamic efficiency may thus be defined as the proportion of the free energy input that is retained in the storage and biomass material produced. Thermodynamic efficiency has been estimated for various biological processes, from mechanical muscular work (~29%; Whipp and Wasserman, 1969) to the growth of microbial cultures (variable, but often approximated as 60%; Xiao and VanBriesen, 2006). The efficiency of conversion of sunlight energy into chemical free energy of plant biomass has been estimated as only a few percent if total annual incident light is compared with harvested biomass, a number which rises to ~30% if red light at non-saturating levels during daytime is considered (Blankenship *et al.*, 2011).

For heterotrophic systems, the available free energy in metabolic input and output has been taken to be the free energy of combustion of substrates and products, since the biologically accessible free energy in metabolites is obtained via oxidation. In practice, the free energies of combustion of biomolecules such as glucose, lipid, protein, etc. are similar to their enthalpies of combustion (Borsook and Huffman, 1938) which are more easily obtained experimentally and are available for biological molecules and biomass (Cordier *et al.*, 1987, and references therein).

Figure 3 shows estimated ECEs of the cells and developing seeds considered in Fig. 1. This comparison again highlights the contribution of light to green seed metabolism, in some



**Fig. 3.** Energy conversion efficiency (ECE) for developing seeds and cell cultures. ECE is defined as the ratio of heat of combustion in biomass components to carbon substrates [ $\Delta H_{\text{comb}}(\text{P})/\Delta H_{\text{comb}}(\text{S})$ ] and is calculated based on literature values for heats of combustion for sugars, polysaccharides, proteins, lipids, and amino acids.

cases raising the energy content (heat of combustion) of the cellular contents above that of the substrates they take up. The fact that ECE values are higher than CCE values highlights the fact that secreted products have lower energy contents (in the case of CO<sub>2</sub> much lower) than the substrates taken up, as well as the fact that central metabolism is quite efficient. Thus heterotrophically grown developing seed tissues of maize and *Brassica* as well as *Arabidopsis* cells achieve ECE values >60%. The energetic demands of transport, turnover of cellular components, and other processes such as signalling necessarily lower the ECE so that values approaching 80% for several other heterotrophic systems may be considered quite high and suggest that in many cases plant cells may be operating at close to optimized energetic efficiencies. Maize endosperm shows an ECE of ~98%, and this is probably explained by the low energetic cost of converting substrate sugars into starch, which forms the large majority of biomass in this tissue. However, even heterotrophically grown *Arabidopsis* cells which do not deposit large amounts of high energy storage compounds achieve ECEs in excess of 70%.

Furthermore, chemical reactions inherently dissipate free energy, with the exception of reactions at equilibrium where there is no net flux. However, it is known that some metabolic reactions carry significant net flux *in vivo* while operating close to equilibrium (such as triose phosphate isomerase and other reactions in glycolysis), and the high energetic efficiency of aerobically growing *E. coli* (Chen *et al.*, 2011) indicates that the non-equilibrium of metabolic systems does not need to impose serious restrictions on ECE. Here the results of <sup>13</sup>C-based MFA have highlighted metabolic reactions that operate near equilibrium (exchange flux  $\gg$  net flux). This is seen in the high values of exchange fluxes for several reactions in published flux maps. The near

**Table 1.** Comparison of carbon and energy conversion efficiency during seed filling in crop and model species

Carbon conversion efficiency (CCE) is defined as the ratio of carbons in biomass components (carbon hydrate, protein, lipid, and cellular organic/amino acids) to feeding carbon sources. The energy conversion efficiency (ECE) is defined as the ratio of heat of combustion in biomass components to carbon substrates consumed [ $\Delta H_{\text{comb}}(P)/\Delta H_{\text{comb}}(S)$ ].

| System   | Substrates                           | CCE   | ECE    |
|--|--------------------------------------|-------|--------|
| <i>Arabidopsis</i> embryos <sup>a</sup> (Lonien and Schwender, 2009)                             | Sucrose+alanine+glutamine            | 80.4% | 96.8%  |
| <i>Camelina</i> embryos, dark <sup>b</sup> (L. Carey and Y. Shachar-Hill, unpublished)           | Alanine+glutamine+sucrose +glucose   | 22.8% | 28.1%  |
| <i>Camelina</i> embryos, 10 $\mu\text{E}^b$ (L. Carey and Y. Shachar-Hill, unpublished)          | Alanine+glutamine+sucrose+glucose    | 40.0% | 49.8%  |
| <i>Camelina</i> embryos, 50 $\mu\text{E}^b$ (L. Carey and Y. Shachar-Hill, unpublished)          | Alaline+glutamine+sucrose+glucose    | 57.0% | 68.9%  |
| <i>Brassica napus</i> embryos, dark <sup>c</sup> (Goffman <i>et al.</i> , 2005)                  | Glucose                              | 60.4% | 79.5%  |
| <i>Brassica napus</i> embryos, 50 $\mu\text{E}^c$ (Goffman <i>et al.</i> , 2005)                 | Glucose                              | 86.1% | 112.6% |
| <i>Brassica napus</i> embryos, 150 $\mu\text{E}^c$ (Goffman <i>et al.</i> , 2005)                | Glucose                              | 94.7% | 124.6% |
| Soybean embryos, 30 $\mu\text{E}^b$ (Allen <i>et al.</i> , 2009b)                                | Glucose+sucrose+glutamine+asparagine | 82.5% | 94.7%  |
| Sunflower embryos <sup>c</sup> (Alonso <i>et al.</i> , 2007a)                                    | Glucose+glutamine                    | 49.5% | 60.4%  |
| Maize embryos <sup>a</sup> (Alonso <i>et al.</i> , 2010)   | Glucose+fructose+glutamine           | 65.5% | 80.4%  |
| Maize endosperm <sup>a</sup> (Alonso <i>et al.</i> , 2011)                                       | Glucose+fructose+ glutamine          | 86.9% | 97.6%  |
| <i>Arabidopsis</i> cell culture low O <sub>2</sub> <sup>a</sup> (Williams <i>et al.</i> , 2008)  | Glucose                              | 65.1% | 73.0%  |
| <i>Arabidopsis</i> cell culture high O <sub>2</sub> <sup>a</sup> (Williams <i>et al.</i> , 2008) | Glucose                              | 63.7% | 73.8%  |
| <i>Arabidopsis</i> cell culture <sup>a</sup> (Masakapalli <i>et al.</i> , 2010)                  | Glucose                              | 60.6% | 72.7%  |
| <i>E. coli</i> aerobic culture <sup>d</sup> (Chen <i>et al.</i> , 2011)                          | Glucose                              | 51.7% | 93.7%  |
| <i>E. coli</i> anaerobic culture <sup>d</sup> (Chen <i>et al.</i> , 2011)                        | Glucose                              | 24.1% | 54.7%  |

<sup>a</sup> CCE was calculated using the reported flux values. The biomass component production rates for ECE calculation were converted from the corresponding carbon effluxes using empirical carbon contents in biomass components: 77% (g g<sup>-1</sup>) in lipid, 52.9% in protein, 40% in glucose, sucrose, fructose, and carbohydrate, 41.1% in glutamine, 36.3% in asparagine, and 40.4% in alanine.

<sup>b</sup> CCE was calculated using the reported <sup>14</sup>C carbon distribution in biomass components. ECE calculation was the same as in <sup>a</sup>.

<sup>c</sup> CCE was reported in the references. The carbon content in biomass components was estimated using the reported biomass composition and the empirical carbon content in <sup>a</sup>. The biomass carbon contents and CCE were used to calculate the amount of feeding substrates. ECE was then calculated as the ratio of heat of combustion in insoluble biomass components to feeding carbon sources.

<sup>d</sup> CCE was calculated from the reported flux map. ECE was calculated from the reported glucose uptake rates (mmol gDW<sup>-1</sup>) and an empirical value of heat of combustion of *E. coli* grown in batch culture on glucose substrate (23.04 kJ g<sup>-1</sup> ash-free biomass) (Cordier *et al.*, 1987).

equilibration of several metabolite sets justifies the lumping together of metabolite pools such as hexose phosphates, triose phosphates, pentose phosphates, and dicarboxylic acids based on the experimentally observed equilibration of labelling patterns (Schwender *et al.*, 2003, 2006; Junker *et al.*, 2007; Allen *et al.*, 2009b).

Interestingly, the ranking of different cells and tissues by ECE for most of the systems shown in Fig. 3 is similar to those in Fig. 1. This reflects the fact that although products differ substantially in their energy contents on a molar or mass basis, the free energy content of both substrates (all the cells considered take up simple sugars and amino acids) and different storage products (carbohydrates, lipids, and proteins) are similar on a per carbon basis. Thus while lipids have higher energy contents per gram and per mole than carbohydrates, they have substantially higher average carbon contents (close to 70% of the mass of lipid is carbon versus 30–40% for carbohydrates and proteins).

Although the relative patterns of ECE values appear similar to those of CCE for most plant systems, ECE has the advantage of being an absolute measure of what is energetically possible. This allows metabolic processes to be compared with other biological and even physicochemical processes such as light capture in photosynthesis or solar panels (Blankenship *et al.*, 2011). Of more metabolic interest is the use of ECE in drawing attention to processes that make, consume, and dissipate available free energy in

metabolism. Here together with MFA, considerations of energy efficiency can help elucidate such questions as what proportion of ATP produced by catabolism is consumed by the production of storage and other biomass components. For example, in developing soybean embryos, the production of protein, carbohydrate, and oil from their metabolic precursors consumed a high proportion (94–98%; Allen *et al.*, 2009b) of potential metabolically derived ATP (the actual ATP production depends on reaction stoichiometries, particularly electron transport–ATPase coupling). In addition, comparing ECE calculated from thermodynamic considerations with the ATP usage efficiency of biomass synthesis (the proportion of ATP produced that it used in biomass synthesis) calculated from flux maps may provide a measure of the effectiveness of energy conservation in the metabolic network. *Escherichia coli* cells cultured under aerobic and anaerobic conditions have ECE values of 93.7% and 55%, respectively, and ATP usage efficiency of 62.9% and 48.9%, suggesting that aerobic cells are more effective in energy conservation.

## Flux balance analysis and efficiency

Flux balance analysis (FBA) provides a computational toolset for analysing efficiencies under the constraints of conservation laws as well as the inherent restrictions

imposed by the limited range of metabolic reactions available to any given organism (Price *et al.*, 2004). The principles and applications of FBA to plants have been reviewed elsewhere (Sweetlove and Ratcliffe, 2011; O'Grady *et al.*, 2012). Here the focus is on its relationship to efficiency, which has not been widely considered, especially for plant systems.

In FBA, conservation of material—including nitrogen and phosphorus as well as carbon—is imposed by tracking these elements and only permitting flux patterns that conserve them. In addition FBA allows a detailed accounting for redox and energy both implicitly by imposing unidirectionality on some reactions and explicitly by accounting for energy and redox cofactors [ATP and NAD(P)(H)]. Thus the range of permissible flux patterns (the feasible solution space of FBA calculations) is constrained to be <100% efficient for both carbon and energy utilization. This extends the range of MFA to metabolic reactions that do not involve carbon, such as mineral nutrient assimilation, transmembrane transport, and energy and redox transduction reactions. FBA can thus serve as a powerful tool for quantifying the contributions of multiple energy-producing and energy-consuming processes (Becker *et al.*, 2007).

As well as allowing the imposition of maximal feasible efficiencies in computing the range of possible flux distributions for any given system, FBA also uses efficiency to guide the prediction of the actual flux distribution. FBA allows the computation of all possible flux distributions that meet the constraints imposed, resulting in a very wide range of feasible solutions. To choose among the feasible solutions and predict the actual flux distribution, it is necessary to identify an 'objective function' to be maximized or minimized. The first and still most popular objective function in FBA predictions of flux maps is maximizing growth (Feist and Palsson, 2010). This means maximizing flux into biomass production and is always expressed relative to the carbon substrate uptake rate. Therefore, FBA with growth rate as its objective function assumes maximal CCE.

While it seems reasonable that maximum growth rates would have been favoured during evolution, particularly by single-celled organisms, there are several reasons why CCE or other efficiencies may not have been optimized. First, the fastest growth rate does not necessarily correspond to the highest efficiency (Schuster *et al.*, 2008). Faster growth may be a result of an increase in resource supply and consequently a global up-regulation of central metabolism at the same or smaller efficiency, as shown in *Arabidopsis* cell culture at higher oxygen levels (Williams *et al.*, 2008). Indeed rapid growth with low CCE may confer a selective advantage by depriving competitors of resources. Secondly, evolution acts over a range of environmental conditions, and may therefore not optimize for maximal growth rate under any one situation. Indeed, culturing microorganisms under controlled conditions for multiple generations (directed evolution) results in substantially improved growth rates without the emergence of new biochemical reactions. These *a priori* doubts are further supported by the fact that FBA predictions of microorganism growth rates and flux patterns

using maximal growth rate per substrate (maximizing CCE) as an objective function are frequently in modest or poor agreement with experiments (Schuetz *et al.*, 2007).

Other objective functions tested in FBA studies include minimizing substrate uptake (at fixed biomass efflux), minimizing energy usage, maximizing ATP yield per unit flux, maximizing biomass yield per total flux, and minimization of reaction steps or total flux (reviewed in Feist and Palsson, 2010). Interestingly, but not to the authors' knowledge explicitly stated, most of these alternative objective functions are also explicitly or implicitly tied to efficiency of one sort or another. Thus energy efficiency is implicit in minimizing energy usage or maximizing ATP yield per unit flux, and the efficient use of material and energy resources devoted to metabolism and growth is maximized when maximizing biomass yield per total flux or minimizing reaction steps or total flux.

Since no one objective function is consistently successful in predicting growth rates, even in simple heterotrophic microorganisms, FBA cannot be used with confidence to predict detailed metabolic phenotypes. However, comparing the actual CCE or ECE with FBA predictions and MFA maps can illuminate the detailed mechanisms by which cells do or do not reach optimal efficiency (Chen *et al.*, 2011). In microorganisms, MFA-derived flux maps have been used with FBA to identify the objective function that best corresponds to metabolism under particular conditions (Feist and Palsson, 2010). These can often be seen to correspond to one sort of efficiency or another. This does not mean that the best fit objective function in any one case is in fact the phenotype or efficiency which natural or anthropogenic selection has acted to optimize. Support for the idea that different efficiencies are selected for in different situations may emerge if future studies show that particular objective functions are frequently found to predict metabolism in particular types of cells or tissues under particular circumstances.

Among plant studies, maximizing biomass production of a maize FBA model produced growth rate predictions that were consistent with literature reports (Saha *et al.*, 2011). FBA predictions of growth rate also agreed with observations of barley seed development when the objective was maximal starch deposition and minimal total flux per carbon substrate taken up (Grafahrend-Belau *et al.*, 2009). This function corresponds to a combination of maximal CCE and minimal allocation of resources to conducting fluxes. In *B. napus* and *Arabidopsis*, predictions of internal fluxes were produced by assuming minimization of input of substrates and/or light per biomass output (a combination of CCE and ECE). The results of the *B. napus* model agreed with flux values obtained from <sup>13</sup>C-based MFA (Hay and Schwender, 2011a). The results of the *Arabidopsis* model were said to agree with experimental observations from various literature sources (Gomes de Oliveira Dal'Molin *et al.*, 2010). In two studies of photosynthetic microorganisms, predictions based on minimizing energy input from light per biomass output (corresponding to maximal ECE) were used to predict growth rates successfully (Shastri and Morgan, 2005; Boyle and Morgan, 2009).



Thus it appears that for developing seeds and photosynthetic microorganisms efficiency in the form of CCE or ECE or a combination of the two is optimized and provides a basis for predicting flux patterns in such plant systems. Evolutionary pressures on microalgae and green seeds that are often heavily shaded, either in natural water bodies or on the plant, may have selected for making the best possible use of light (ECE). For seeds it is known that reproductive success in nature is higher for plants producing more and larger seeds (Black, 1958; Howe and Richter, 1982; Armstrong and Westoby, 1993; Vaughton and Ramsey, 1998) and that agricultural breeding selection has targeted the same traits. Although these pressures are on biomass accumulation rates not on efficiencies, when combined with limitations of light, they would be expected to lead to optimized energy and carbon use efficiencies.

If these selection pressures are not dominant in defining metabolic flux maps, one would expect to see cases where neither CCE nor ECE predicts flux maps. Interestingly, in a study of cultured heterotrophically grown *Arabidopsis* cells exposed to normal versus oxidative stress conditions (Williams *et al.*, 2010), comparison of FBA and MFA maps point to minimization of total flux as the objective function that yielded computed fluxes in good agreement with measured ones. It is therefore of interest to consider when one might expect agreement between fluxes predicted by maximizing efficiency and measured fluxes while also bearing in mind that only a small number of studies have been conducted where high quality MFA flux maps have been compared with FBA predictions in plant systems.

Growing pollen tubes may be one case where CCE and/or ECE may have been under strong selection pressure. Here selection is based on reaching and fertilizing an egg as quickly as possible, as there are frequently many more pollen than eggs. Therefore, speed not efficiency is selected and there is no need for high efficiency if internal stores or external C are sufficient. Geometrically based estimates of pollen grain contents versus biomass needed to produce a long pollen tube (not shown) indicate that internal stores are insufficient for growing pollen tubes to reach the eggs. Therefore, one may expect that efficiency of storage utilization for biomass production (CCE) should be selected since exogenous C will not always be available in sufficient amounts. In roots too it may be expected that CCE is optimized since efficient soil exploration with minimal resource allocation should confer a competitive advantage. The root tip is mainly devoted to making more root in rapid heterotrophic growth, and several MFA studies have been directed at mapping central metabolic fluxes in this tissue (Dieuaide-Noubhani *et al.*, 1995; Alonso *et al.*, 2007b). The finding of high rates of futile cycling in root tips has been questioned (Kruger and Ratcliffe, 2009), but if verified would contradict the idea that maximized CCE is likely to predict flux patterns correctly in this tissue. Another case where CCE should be strongly favoured is during seed germination. Germination of seeds and the non-photosynthetic growth of seedlings involve the conversion of storage materials in new biomass. The efficiency of this conversion will determine the

maximal growth of seedlings before they must become autotrophic. In many natural settings, shading of young seedlings restricts photosynthesis, and their success or failure depends on how much they can grow using their storage reserves. Under these conditions, higher CCE confers a fitness advantage and one would expect that this would be high and perhaps not very variable. In species whose habitat does not impose such limits, and in crops bred for higher seed size than circumstances require, one might suppose that efficiency would be lower and more variable. It would be of interest to determine CCE and fluxes during germination in varieties of a long-domesticated crop species in comparison with those of wild relatives.

## Conclusion

Flux analysis studies have illuminated the basis of carbon and energy conversion efficiencies in plant cells and tissues. Efficient use of low levels of light to conserve carbon is associated with several unusual features of central metabolic flux in developing green seeds. Although not emphasized, constraints-based flux balance analysis relies on assumptions about maximized efficiency in the form of objective functions. Comparisons in a few cases have been made between flux maps measured by <sup>13</sup>C-based MFA and those predicted by FBA assuming one or more maximal efficiency parameters. These studies suggest that developing plant seeds and photoautotrophic microorganisms may indeed have patterns of metabolic flux that maximize efficiency. *Arabidopsis* cells cultured in liquid media even demonstrated global regulation of metabolism in response to the change of oxygen supply to maintain carbon efficiency. MFA and FBA are synergistic toolsets for uncovering and explaining the metabolic basis of efficiencies and inefficiencies in plant systems.

## Supplementary data

Supplementary data are available at *JXB* online.

**Supplementary Materials and methods.** *Camelina* culture and CCE calculation.

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