Capturing Metabolite Channeling in Metabolic Flux Phenotypes[1][W]

Thomas C.R. Williams, Lee J. Sweetlove, and R. George Ratcliffe*

Departamento de Biologia Vegetal, Universidade Federal de Viçosa, 36570–000 Viçosa, Minas Gerais, Brazil (T.C.R.W.); and Department of Plant Sciences, University of Oxford, Oxford OX1 3RB, United Kingdom (L.J.S., R.G.R.)

Metabolite channeling, also known as substrate channeling, the process by which intermediates in metabolic pathways are passed from enzyme to enzyme without equilibration with the cellular medium, is thought to be important in the organization and regulation of metabolism (Agius and Sherratt, 1997). The process is difficult to study noninvasively, particularly in the highly connected network of central metabolism, but since channeling can provide alternative pathways its occurrence may leave a footprint in the ^{13}C-labeling datasets that are now used for the steady-state metabolic flux analysis (MFA) of central metabolism in heterotrophic and mixotrophic plant tissues (Ratcliffe and Shachar-Hill, 2005, 2006; Libourel and Shachar-Hill, 2008; Schwender, 2008; Allen et al., 2009; Kruger and Ratcliffe, 2009). It is shown here, using existing ^{13}C-labeling data for Arabidopsis (*Arabidopsis thaliana*) cell suspensions (Williams et al., 2008, 2010), that well-defined channeled fluxes can be detected in glycolysis and the tricarboxylic acid (TCA) cycle using steady-state MFA.

Metabolite channeling is known to occur in both primary and secondary plant metabolism (Winkel, 2004; Jørgensen et al., 2005; Graham et al., 2007). Despite this, there has been no explicit consideration of the phenomenon, either in plants or in other organisms, in the models that are used in steady-state MFA to deduce metabolic fluxes through the network of central carbon metabolism. In fact these models can be readily extended to allow for the impact of channeling on the redistribution of label in a steady-state MFA experiment, and two possibilities are of immediate interest (Fig. 1). First, channeling may prevent the randomization of orientation that would normally occur when a symmetric metabolite equilibrates with the bulk medium between enzyme-catalyzed reactions. If the symmetric metabolite is asymmetrically labeled, then orientation conserved transfer of label in the channeled pathway will lead to a different labeling pattern in the downstream metabolites from the one expected for an unchanneled system (Fig. 1A). For example asymmetrically labeled fumarate might be channeled through fumarase, leading to asymmetric labeling of malate and Asp (Fig. 1B; Sumegi et al., 1993); whereas these products of fumarate would be symmetrically labeled in the absence of channeling. Second, and more generally, channeling might provide inequivalent routes for the labeling of an intermediate, leading to a change in the labeling pattern of a downstream metabolite (Fig. 1C). For example a channeled glycolytic pathway could bypass input of label from the pentose phosphate pathway at the level of triose phosphate, altering the isotopomer composition of metabolites derived from phospho*enol*pyruvate (Fig. 1D).

The impact of these two channeling schemes on the redistribution of label in a steady-state labeling experiment was explored using the MFA software ^{13}C-FLUX (Wiechert et al., 2001). Models were established for the two network structures (Supplemental File S1) and the extent to which the channeling flux could be deduced from the labeling data provided was investigated. These simulations showed that the redistribution of the label was sensitive to the presence of the channeling and that its extent could be quantified by steady-state MFA (Supplemental Table S1). This encouraging result suggested that it would be worthwhile to consider the impact of introducing channeling steps into the metabolic model that has been used to deduce flux maps of heterotrophic metabolism in cell suspensions of Arabidopsis (Williams et al., 2008, 2010).

As a first step, two previously published datasets from [1-^{13}C]Glc labeling experiments, one based on ^{13}C-NMR measurements (Williams et al., 2008) and the other based on gas chromatography-mass spectrometry and ^{1}H-NMR measurements (Williams et al., 2010), were reanalyzed simultaneously to generate a flux map of primary metabolism. Supplemental File S2 defines the model of the unchanneled network and Supplemental File S3 provides a commentary on the fitting procedure. The fluxes (Supplemental File S4) provide a good explanation of the experimental data and the model passes the χ^2 test for goodness of fit (Supple-

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* Corresponding author; e-mail george.ratcliffe@plants.ox.ac.uk.

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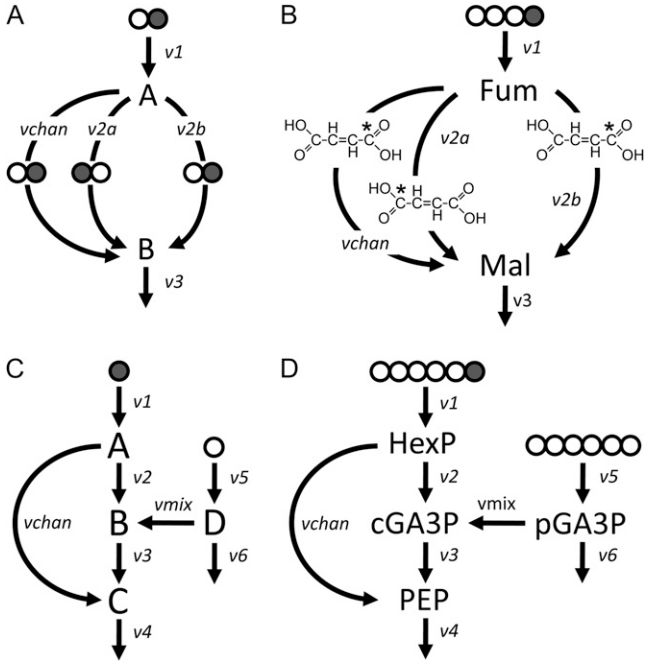

Figure 1. Examples of network structures in which a channel between two metabolites provides an alternative route to a conventional pathway. A, Orientation conserved metabolism of a symmetric molecule containing two carbon atoms (A) running in parallel with a pathway in which A is released into the bulk medium before conversion to B. If A is derived from an asymmetrically labeled precursor then the orientation of the labeling may be preserved during the conversion of A to B in the channel; whereas if A is released into the bulk medium before conversion to B, the position of the label will be randomized. It follows that the observed labeling in B, and in metabolites derived from it, will reflect the relative fluxes through the channeled (*vchan*) and unchanneled (*v2a* and *v2b*) pathways. B, Example of orientation conserved transfer that might occur in the TCA cycle: The asymmetry in the labeling (*) of fumarate is maintained in the channeled pathway but lost in the unchanneled pathway. C, Scheme in which a channeled flux provides a direct route between molecules A and C, avoiding potential changes in the labeling of B and C via the contribution of *vmix*. D, Example of a metabolic bypass that might occur in glycolysis: Synthesis of phospho*enol*pyruvate via the channel bypasses the contribution of the plastidic triose phosphate pool (pGA3P) to the labeling of the cytosolic triose phosphate pool (cGA3P). In each scheme, the labeling of the products of the competing pathways depends on the relative flux through the two pathways. White circles = unlabeled carbon atoms; shaded circles = labeled carbon atoms.

mental Table S2). The fluxes are similar in most respects to those published earlier, and thus offer a suitable platform for testing the impact of introducing putative channels in several areas of central metabolism.

One area of the network in which these effects could be important is carbohydrate oxidation, since a partially channeled glycolytic pathway is known to be associated with the outer mitochondrial membrane in Arabidopsis (Giegé et al., 2003; Graham et al., 2007). Adding a bypass of the triose phosphate node to the model, to allow channeling between the cytosolic hexose phosphate pool and phospho*enol*pyruvate (Fig. 1D; Supplemental Fig. S1), led to a set of solutions with a nonzero value for the channeling flux (Supplemental Table S2). Optimal solutions were obtained with and without the channeling flux (Supplemental File S4) and the channeled flux was deduced to be 2.4 mmol d^{-1} L culture^{-1}. Critically, determination of the 95% confidence interval (Antoniewicz et al., 2006) showed the channeled flux to be well defined and significantly different from zero (Supplemental Fig. S3). While the impact on the adjacent fluxes in the network was small (Supplemental File S4), reflecting the small value of the flux through the channel, the model with channeling provides a better description of the system because the fit of the model is significantly improved when the additional pathway carries a flux that is significantly different from zero.

This result demonstrates that steady-state MFA can identify known instances of metabolite channeling, and it suggests that it may also be used to detect metabolite channels that have not been identified previously in the central metabolic network. This was tested by modifying the Arabidopsis model to include a metabolite channel from the cytosolic hexose phosphate pool to oxaloacetate. Such a channel, if it existed, could support the anaplerotic provision of carbon skeletons for the TCA cycle, and might therefore provide an additional function for the glycolytic enzymes associated with the outer membrane of plant mitochondria (Giegé et al., 2003; Graham et al., 2007). A small nonzero flux of 1.8 mmol d^{-1} L culture^{-1} was obtained for the channel (Fig. 2, A and B; Supplemental File S4), with 95% confidence limits of 1.2 and 2.5 mmol d^{-1} L culture^{-1}, and in this model the pathway provided all the anaplerotic flux into the TCA cycle. The revised model again provided a good explanation of the data and a better description of the system (Supplemental Fig. S2B; Supplemental Table S3).

Channeling through the reactions of the TCA cycle has been detected in several organisms and tissues (Srere et al., 1997), but the extent of channeling in the plant TCA cycle is unknown. To address this, steady-state flux solutions for the Arabidopsis cells were obtained for a series of flux models that included channeling between pairs of TCA cycle metabolites. During repeated bootstrap Monte Carlo fitting of the models some of these channels always carried zero flux (Supplemental Table S2), whereas others—notably the channels between fumarate and citrate (Fig. 2, C and D; Supplemental Fig. S1) and between malate and citrate—were almost always active and responsible for the majority of the flux between these pairs of metabolites (Supplemental Table S2; Supplemental File S4). The optimal solutions for these two channeled fluxes were well determined (Table I), and introducing a channeled flux between fumarate and citrate had a significant impact on the flux map for the TCA cycle (Fig. 2, C and D).

These results have two major implications for the analysis of central carbon metabolism. First, metabolite channeling can be a significant factor in determining the steady-state redistribution of isotopic label in

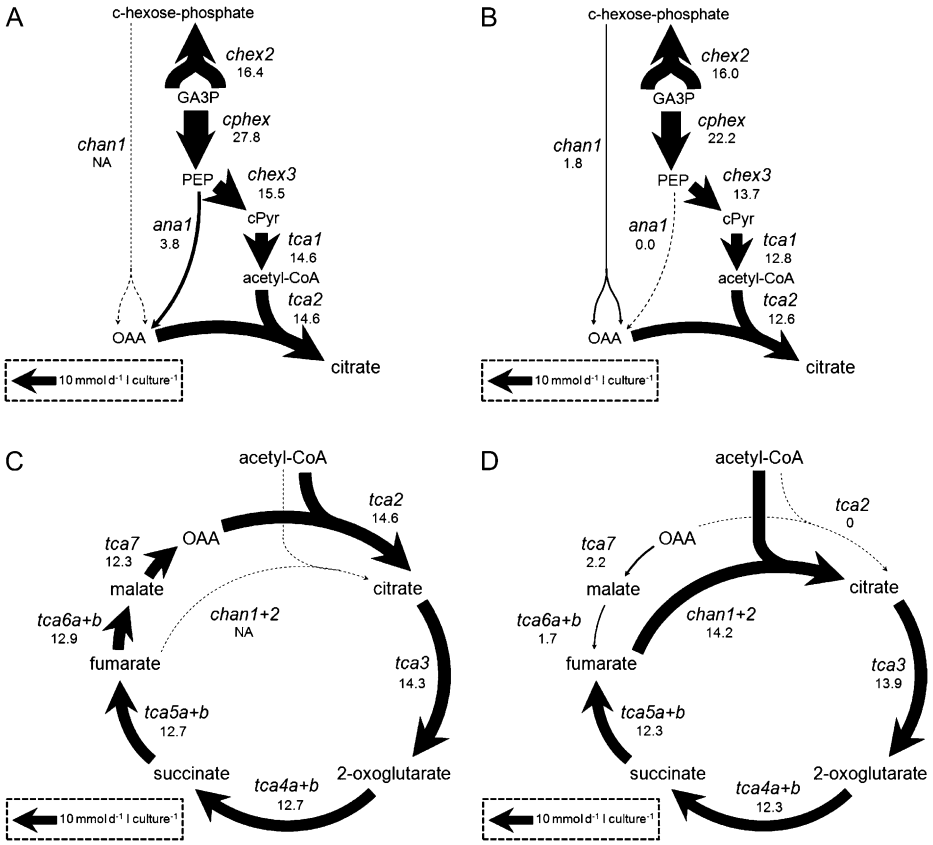

Figure 2. Flux maps showing the effect of glycolytic and TCA cycle metabolic bypasses. A, Glycolysis and entry into the TCA cycle. B, Glycolysis and entry into the TCA cycle, with the option of channeling from hexose phosphate to oxaloacetate. C, TCA cycle. D, TCA cycle with the option of channeling from fumarate to citrate. Flux names (*cphex*, *tca5a + b*, etc.) are defined in the model (Supplemental File S2; Supplemental Fig. S1). The width and direction of the arrows represent flux in mmol d^{-1} L culture^{-1}. Exchange fluxes and fluxes to biomass components are not illustrated. Complete flux solutions are given in Supplemental File S4, and the complete model is illustrated in Supplemental Figure S1.

the network of central carbon metabolism, and this needs to be considered in constructing the models used for steady-state MFA. High-quality labeling data, coupled with the simultaneous analysis of experiments with different labeled precursors (Schwender et al., 2006; Libourel et al., 2007; Masakapalli et al., 2010), is improving the definition of the flux maps produced by steady-state MFA, and it is imperative to base these maps on realistic metabolic models that capture the likely features of the network. Second, steady-state MFA now offers a general noninvasive strategy for the detection of metabolite channeling in the central metabolic network that complements the existing methods, many of which are invasive. Ultimately the success of the method will depend on the structure and properties of the whole network, as well as on the extent to which the labeling strategy is able to report on the fluxes of interest. Thus it is to be expected that further increases in the sophistication of the models used for the analysis of heterotrophic metabolism by steady-state MFA, perhaps to include the cytosolic bypasses of TCA cycle enzymes (Sweetlove et al., 2010) or to capture the subcellular compartmentation of carbohydrate oxidation (Masakapalli et al.,

Table I. *Fluxes through putative TCA cycle channels determined using steady-state MFA*

Multiple fits were carried out for models including each of the putative channels indicated in the Table (for details, see Supplemental Table S2) and 95% confidence limits (given in parentheses) were determined for the channeling flux. Fluxes are given as both net and forward/reverse values in mmol d^{-1} L^{-1} culture. Flux names (tca5, etc.) are defined in the model (Supplemental File S2; Supplemental Fig. S1) and the complete flux solutions are provided in Supplemental File S4. 2-OG, 2-Oxoglutarate; MDH, malate dehydrogenase; SDH, succinate dehydrogenase.

Channel	Net Fluxes				Forward/Reverse	
	Channelling Flux Value (95% Limits)	SDH (tca5_net)	Fumarase (tca6_net)	MDH (tca7_net)	Fumarase (tca6_Xch)	MDH (tca7_Xch)
	mmol d^{-1} L culture^{-1}					
None	–	12.66	12.90	12.29	2,244/2,231	1,128/1,115
2-OG → citrate	0.0 (0, 1.6)	12.67	12.92	12.30	2,244/2,231	1,128/1,115
Succinate → citrate	0.0 (0, 1.5)	12.35	12.59	12.05	2,244/2,231	1,128/1,115
Fumarate → citrate	14.2 (9.5, 14.2)	12.27	−1.67	−2.22	241/243	1,115/1,118
Malate → citrate	14.5 (10.6, 14.5)	12.59	12.83	−2.31	203/190	1,115/1,118

2010), may have further implications for the characterization of channeled fluxes that bypass unchanneled pathways.

In conclusion, steady-state MFA offers a viable method for quantifying channeled fluxes in the network of central carbon metabolism, and the detection of significant channeling in both glycolysis and the TCA cycle indicates where functionally important associations between enzymes might be found. This is important for three reasons. First, metabolic channeling is an elusive phenomenon that has proved difficult to characterize in central metabolism and an approach that reveals channeling in the context of a network-wide quantitative analysis of the redistribution of isotope label may be inherently more reliable than a targeted approach that ignores the wider impact of the network. Second, steady-state MFA aims to capture metabolic phenotypes, and flux maps that more accurately represent the metabolic activity of a cell or tissue are likely to be more useful. Finally, improved knowledge of the extent of metabolic channeling should lead to an appreciation of its physiological significance, and to a greater understanding of its potential role in regulating the fluxes in primary metabolism.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Metabolic model for Arabidopsis.

Supplemental Figure S2. Measured and simulated isotopomer abundances.

Supplemental Figure S3. Determination of 95% confidence limits for a channeled flux.

Supplemental Table S1. Modeling of the channeling flux in Figure 1C.

Supplemental Table S2. Modeling of channeling fluxes in Arabidopsis.

Supplemental Table S3. Residual and $\chi 2$ test results for Arabidopsis models.

Supplemental File S1. Models corresponding to Figure 1 (Excel format).

Supplemental File S2. Model for central carbon metabolism in heterotrophic Arabidopsis cells (Excel format).

Supplemental File S3. Commentary on the models and fitting procedure.

Supplemental File S4. ^{13}C-FLUX solutions for metabolic models of heterotrophic Arabidopsis cells with and without channeling.

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