New Phytologist

Review

Tansley insight

Isotopically nonstationary metabolic flux analysis of plants: recent progress and future opportunities

Author for correspondence:
Doug K. Allen
Email: doug.allen@ars.usda.gov

Received: *4 February 2024*
Accepted: *6 March 2024*

Somnath Koley[1], Poonam Jyoti[1], Maneesh Lingwan[1] and Doug K. Allen[1,2]

[1]Donald Danforth Plant Science Center, 975 North Warson Road, St Louis, MO 63132, USA; [2]United States Department of Agriculture, Agriculture Research Service, 975 North Warson Road, St Louis, MO 63132, USA

Contents

New Phytologist (2024)
doi: 10.1111/nph.19734

Key words: biochemical pathways, carbon partitioning, central carbon metabolism, isotopic labeling, metabolic flux analysis, metabolic modeling, plant metabolism, systems biology.

Summary

Metabolic flux analysis (MFA) is a valuable tool for quantifying cellular phenotypes and to guide plant metabolic engineering. By introducing stable isotopic tracers and employing mathematical models, MFA can quantify the rates of metabolic reactions through biochemical pathways. Recent applications of isotopically nonstationary MFA (INST-MFA) to plants have elucidated nonintuitive metabolism in leaves under optimal and stress conditions, described coupled fluxes for fast-growing algae, and produced a synergistic multi-organ flux map that is a first in MFA for any biological system. These insights could not be elucidated through other approaches and show the potential of INST-MFA to correct an oversimplified understanding of plant metabolism.

I. Introduction

The partitioning of resources through metabolism in plants enables growth, confers resilience to stress, and establishes crop yield. Therefore, a quantitative understanding of plant metabolism is crucial to sustainably feed and fuel a growing population on less land and in future changing climates. Contemporary approaches that assess cellular processes to improve plant productivity rely on system biology tools including genomics, transcriptomics, proteomics, and metabolomics. These tools provide an inventory of cellular components and can suggest regulation at different levels, though none are intended to be a proxy for flux. Thus, system biology models attempting to link omics frequently yield discordant results (Fernie & Stitt, 2012; Schwender *et al.*, 2014). The discrepancies arise in part because omics tools measure a concentration or level at a snapshot in time and do not inherently distinguish production of metabolically active molecules from remnants of a prior metabolic state. Metabolic flux analysis (MFA) addresses this gap. Fluxes are strictly related to one another by mass conservation principles, and their measurement provides dynamic information about the active flow of atoms through metabolic pathways that quantitatively define cellular operation and result in the observed plant phenotype.

The internal fluxes resulting from MFA often cannot be measured using alternative methods and provide a comprehensive picture that serves as a basis for defining and validating the metabolic objectives of a network through subsequent genetic studies. For example, the role of malic enzyme and isocitrate dehydrogenase in fatty acid production in oilseeds (reviewed in Allen *et al.*, 2015) indicated unexpected flux patterns that could contribute to biotechnologically relevant phenotypes. Some of these ideas were recently validated by a genetic study with altered subcellular levels of malic enzyme (Morley *et al.*, 2023; Schwender, 2023). Studies surveying the impact of abiotic stress, including altered light intensity (Ma *et al.*, 2014; Medeiros *et al.*, 2022; Treves *et al.*, 2022), temperature (Sharkey *et al.*, 2020), or nutrient status (Allen & Young, 2013; Masakapalli *et al.*, 2013, 2014; Zhang *et al.*, 2018), and descriptions of unanticipated storage reserve remobilization in engineered plants (Chu *et al.*, 2022) hold potential significance for crop production given expected shifts in climate and agricultural conditions. Flux studies can also provide a fundamental assessment of the crosstalk between complementary biosynthetic pathways, such as spatially distinct terpene production (Koley *et al.*, 2020) or the role of steps in oxidative pentose phosphate pathway relative to glycolysis in seeds (Carey *et al.*, 2020) or to Calvin–Benson cycle (CBC) in leaves (Xu *et al.*, 2021, 2022). These examples indicate that portrayals of plant metabolism as an extension of heavily studied systems including *Escherichia coli* or *Arabidopsis thaliana* are oversimplified and do not reflect the metabolic diversity among species (Stitt *et al.*, 2021; Clapero *et al.*, 2023). Metabolic flux analysis studies emphasize central metabolism, which is the basis for biosynthesis of all biomolecules, is pliable and context-specific, varies within organs and tissues, and accommodates perturbations in potentially unanticipated ways (Allen, 2016). Such studies provide a foundational ground-truthing for conclusions derived from other omics or scientific investigations and an understanding of metabolic network operation that can be tested through genetic alterations or changes in the environment.

II. Basic considerations for metabolic flux analysis

Metabolic fluxes are inferred by tracking the movement of isotopes through bond breaking and reforming reactions (Kruger & Ratcliffe, 2015). The use of isotopes to assess flux is not new and was the primary approach to elucidating metabolic pathways before advances in mutant generation. Historical descriptions of photosynthetic, central, and lipid metabolic pathways are based on isotope tracing (Allen *et al.*, 2015); however, MFA studies use computational modeling to deduce network fluxes that establish the redistribution of label. Fig. 1 summarizes the basic steps including choosing an isotope or combination that are incorporated over spans of time most relevant to the metabolism of interest and quantifying labeled and unlabeled atoms within metabolites by mass spectrometry and nuclear magnetic resonance (Allen & Ratcliffe, 2009). Then, the tracer description, metabolic network, measured rates, and quantified labeling in metabolites are used within a computational model that optimizes fluxes to minimize the error between experimental and *in silico* measurements across the entire network as reviewed elsewhere (Kruger *et al.*, 2012; Antoniewicz, 2013).

III. Transformative advances enabled MFA in autotrophs

Labeling of metabolites can be measured once they are isotopically unchanging (i.e. steady-state-MFA, or SS-MFA) or during the transient incorporation of isotope over time (i.e. isotopically nonstationary, or INST-MFA) (Fig. 2). Among plant tissues, long metabolic steady states required for SS-MFA are the exception and exclude diurnally changing photosynthetic tissues. Further, autotrophic metabolism relies on a single source of carbon (CO_2) that would entirely label metabolites at isotopic steady state and be uninformative (Roscher *et al.*, 2000; Shastri & Morgan, 2007). The development of INST-MFA software tools (Matsuda *et al.*, 2021; Rahim *et al.*, 2022; Borah Slater *et al.*, 2023; Wu *et al.*, 2023) and the capacity to measure labeling in phosphorylated intermediates of central metabolism (Arrivault *et al.*, 2015; Koley *et al.*, 2022a) with electrospray ionization-MS (ESI-MS) enabled flux studies in systems with short metabolic steady states (minutes–hours) including those with photosynthetic CO_2 assimilation (Szecowka *et al.*, 2013; Ma *et al.*, 2014).

IV. Insights established from INST-MFA

The potential for INST-MFA is highlighted here, by five studies that describe novel, unanticipated insights into central metabolism. Isotopically nonstationary MFA in plants initially focused on the response of photoautotrophic fluxes to high-light acclimation in leaves (Ma *et al.*, 2014). The flux maps identified differences in subcellular fluxes and 'inactive' metabolite pools that diluted measured labeling as a result of cellular heterogeneity and slow pool turnover (e.g. serine, UDP-glucose). Further, the study indicated high-light acclimation resulted in more biomass that was produced less efficiently due to increased photorespiratory CO_2 release. In essence, long-term high-light exposure increased CBC carboxylation and a greater drawdown on CO_2, which was incompletely accommodated by changes in leaf architecture (Terashima *et al.*, 2011) and resulted in proportionally more photorespiration (Fig. 3a) as part of the acclimation strategy.

Isotopically nonstationary MFA has recently uncovered novel aspects of hexose phosphate metabolism (Xu *et al.*, 2021, 2022) in Camelina leaves. The seminal observation of incompletely labeled CBC metabolites even after prolonged exposure to $^{13}CO_2$ in photosynthetic leaves was explained by recycling stored carbohydrate pools. Basically, unlabeled sucrose was converted to glucose 6-phosphate (G6P), which entered CBC via oxidative conversion to pentose phosphates (G6P shunt) and diluted the labeling of CBC intermediates. The authors estimated that possibly 93% of non-photorespiratory CO_2 released, that is day respiration, is attributable to the G6P shunt and not mitochondrial decarboxylation through the TCA cycle or fatty acid biosynthesis (Xu *et al.*, 2021) (Fig. 3b). Historically perceived futile cycles, such as potentially counterproductive fluxes through oxidative and reductive (CBC) steps, may actually help subvert stress (Sharkey

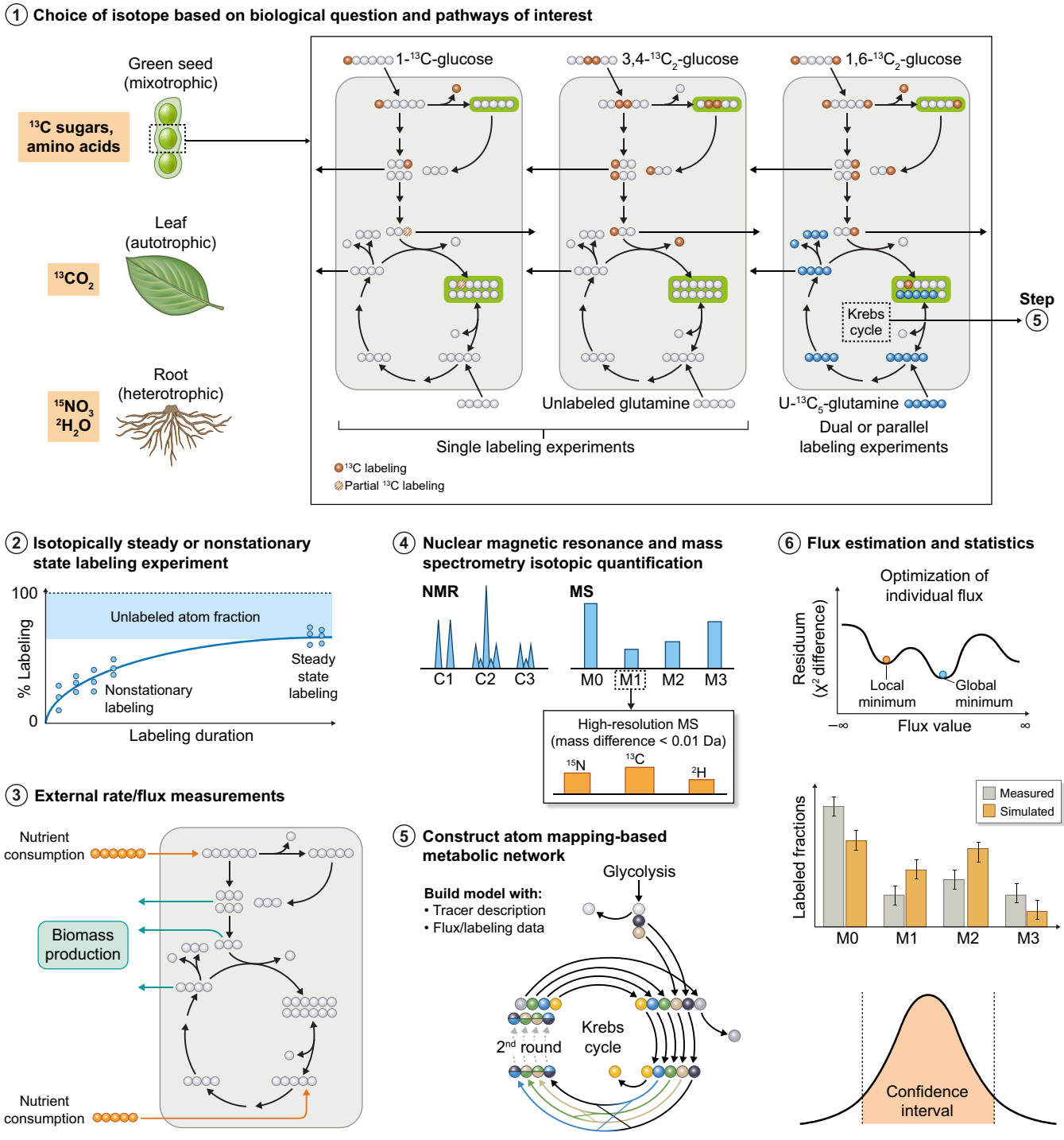

Fig. 1 Key steps of metabolic flux analysis. Step 1: Select an isotopic tracer based on the biological question. For instance, ^{13}C-labeled substrates (single or dual/parallel labeling) are often used to study carbon flux in mixotrophic systems, while $^{13}CO_2$ is used in autotrophic systems. ^{15}N or ^{2}H are more applicable for roots that take up nutrients and water. Step 2: Determine the type of labeling experiment. For systems with long metabolic steady states, labeled substrates are continuously supplied until an isotopic steady state is achieved before sampling. Nonstationary labeling conditions involve multiple sampling times to assess the labeling dynamics. Nonstationary labeling is required when metabolic steady states are short-lived. Step 3: Measure external rates of substrate consumption or product formation such as the production of biomass. Step 4: Use nuclear magnetic resonance, and/or mass spectrometry (MS) and high-resolution-MS to quantify labeling in metabolites. Step 5: Construct a metabolic network that represents the atom transitions between biochemical reactions. Step 6: Estimate flux parameters computationally using metabolic flux analysis platforms that minimize the difference between experimental and *in silico* data through regression. Repeat the analysis with different initial parameter estimates to maximize the probability of obtaining global optimal values. Calculate confidence intervals or perform other statistical evaluations to assess the certainty in fluxes. Iteratively adjust the network to best represent the biology and repeat the evaluation until satisfied.

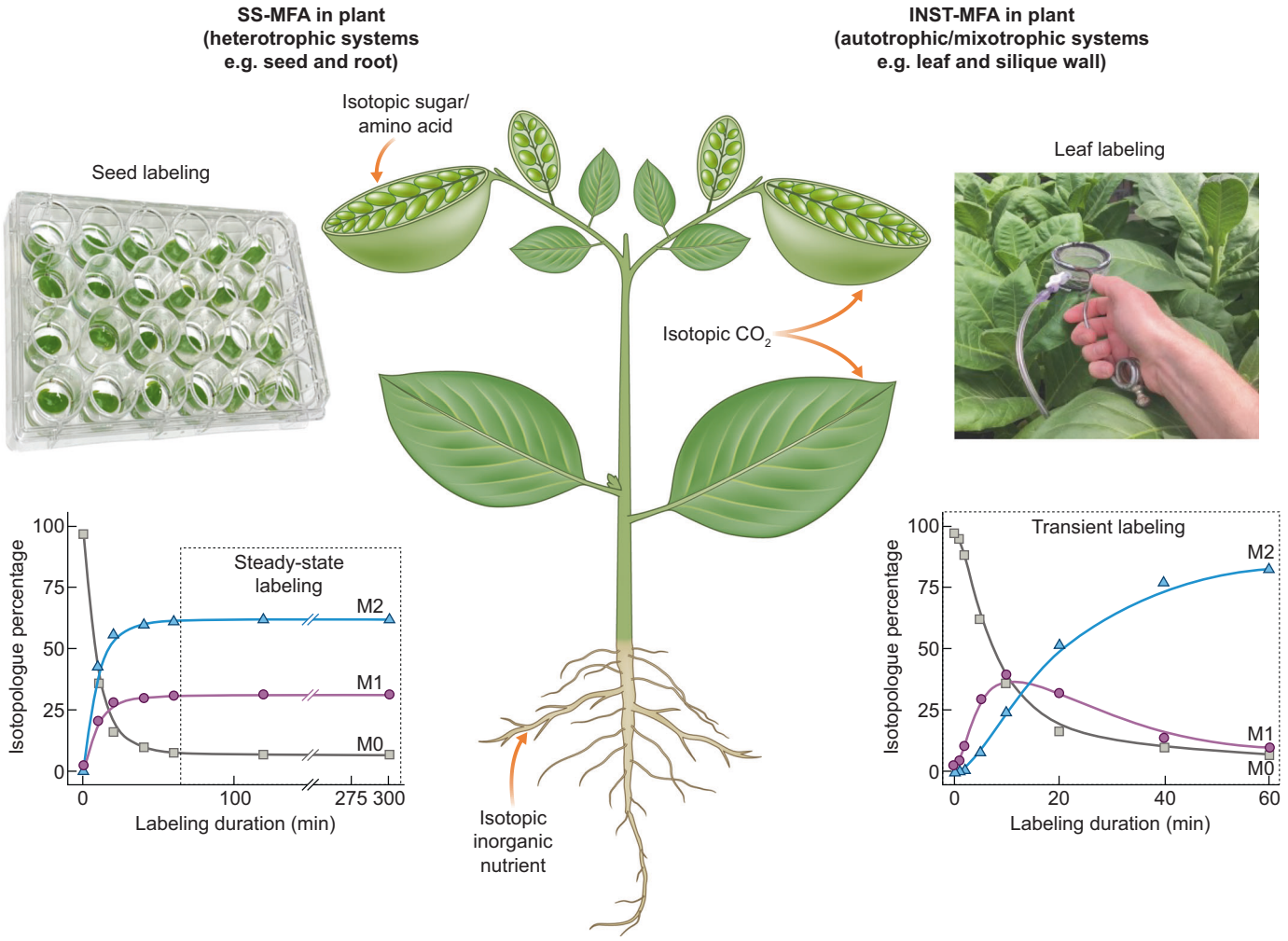

Fig. 2 Choices in labeling and metabolic flux analysis (MFA) approach. Differences in isotopically steady or transient labeling that are used with steady state MFA (SS-MFA) or isotopically nonstationary MFA (INST-MFA), respectively, and commonly associated with heterotrophic or autotrophic metabolism.

et al., 2020), and stabilize substrate availability for the CBC, increasing responsiveness to dynamic field conditions (Allen & Young, 2020; Medeiros *et al.*, 2022) or altered CO_2 (Wieloch *et al.*, 2022). Other pathways that historically were simplistically described as cyclic and wasteful, such as photorespiration, are now known not be closed loops (Busch *et al.*, 2018; Fu *et al.*, 2023) and provide carbon for other aspects of metabolism. Fu *et al.* (2023) showed a remarkable 23–41% of serine flux leaves photorespiration, although the use of metabolites exiting the pathway remains a point of interest.

To date, one of the most comprehensive INST-MFA studies involved eight labeling experiments including $^{13}CO_2$, U-$^{13}C_6$, 1-^{13}C, 6-^{13}C and 1,6-$^{13}C_2$ glucose, U-$^{13}C_5$ glutamine, U-$^{13}C_4$ malate, and U-$^{13}C_3$ alanine, and resulted in a multi-organ flux map that was the first of this kind for any biological system, plant, or otherwise (Koley *et al.*, 2022b) describing the synergy between reproductive tissues in Camelina. Silique-based photosynthesis contributed 33–45% of all carbon within the seed (Fig. 3c). Further, recovery of seed-respired CO_2 by silique walls increased carbon use efficiency from 63% to 70% when reproductive tissues were considered in combination. Shading experiments of leaves or siliques indicated a significant latent capacity for greater seed yield. Perhaps this untapped productivity is an adaptation to mitigate the fluctuating environmental effects plants encounter in the field. Green siliques have unencumbered access to sunlight and their close proximal location to developing seeds that minimizes sucrose translocation and provides a developmentally 'just-in-time' delivery of photoassimilates is potentially an architectural optimum designed by nature. Leaf photosynthate can be allocated to roots, stems, and other sinks of the plant to produce hardy crops.

Changes in photoassimilate partitioning were considered in engineered tobacco. Plant leaves generally produce starch during the day that is turned over at night, although some accumulate starch in their leaves over development. Isotopically nonstationary MFA (Chu *et al.*, 2022) of genetically altered tobacco leaves, which were engineered to produce over 30% lipid (Vanhercke *et al.*, 2017), indicated changes in metabolism that accompanied a switch from starch to lipid production. Flux maps quantified enhanced malic enzyme activity to provide carbon and reducing

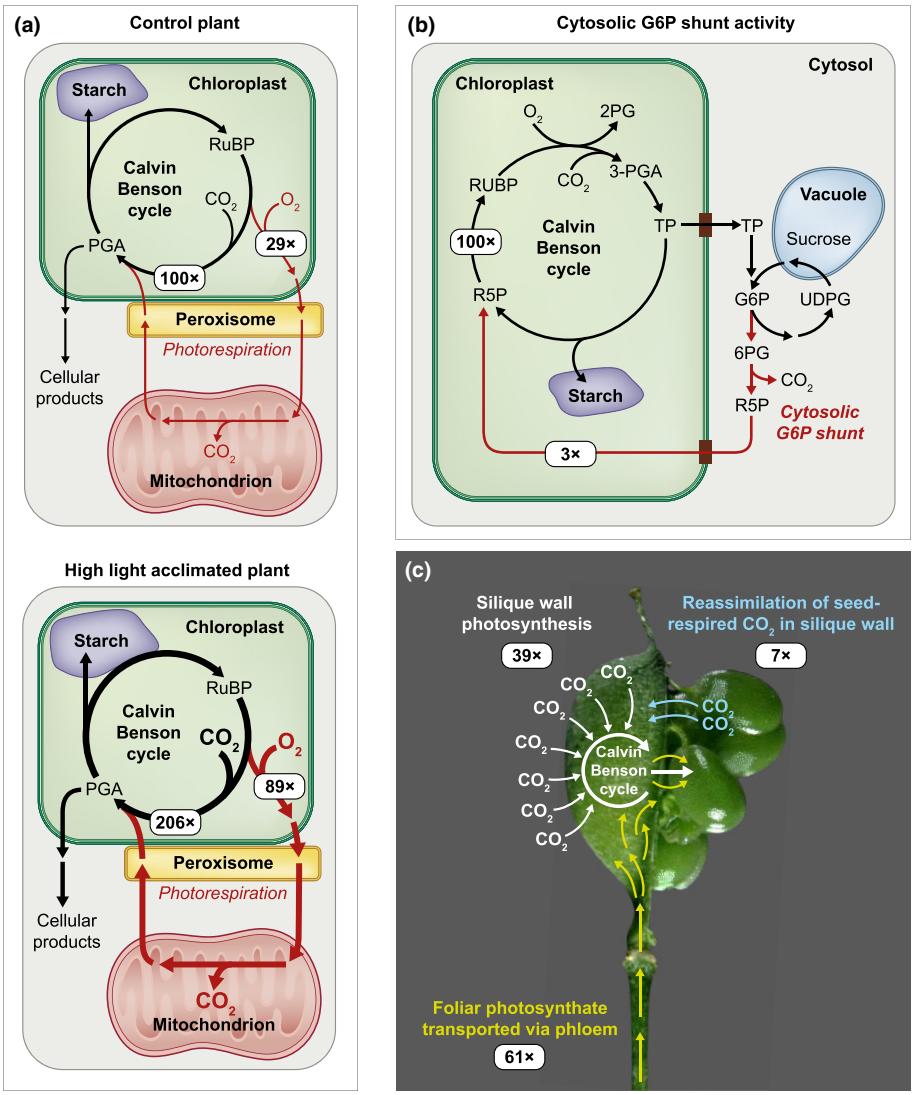

Fig. 3 Flux phenotypes in plants unveiled through isotopically nonstationary MFA (INST-MFA). (a) Flux analysis (Ma *et al*., 2014) describing the adaptation of photosynthetic metabolism under high light in *Arabidopsis thaliana*. Photosynthetic CO_2 fixation was increased under high light, resulting in an increase in biomass; however, photorespiration, a carbon-inefficient pathway, was also increased. (b) A flux study in *Camelina sativa* (Xu *et al*., 2022) indicated the presence of the G6P shunt along with the Calvin–Benson cycle (CBC) in leaves. The shunt supplies carbon to CBC from large vacuolar and cytosolic sucrose pool. (c) A multi-tissue flux analysis in *C. sativa* (Koley *et al*., 2022b) quantified the important photosynthetic role of a non-foliar tissue, the silique wall, in seed metabolism and indicated that silique walls and seeds act synergistically to create a more carbon-efficient reproductive structure. 2PG, 2-phosphoglycolate; 6PG, 6-phosphogluconate/6 phosphogluconolactone; CO_2, carbon dioxide; G6P, glucose 6-phosphate; O_2, oxygen; PGA, phosphoglyceric acid; R5P, ribose 5-phosphate; RuBP, ribulose 1,5-bisphosphate; TP, triose phosphate.

equivalents for fatty acid biosynthesis and increased Rubisco carboxylase activity. Unlike other crops, tobacco was domesticated for productive leaves rather than seeds. Thus, one implication is that if leaves of some plants can act as a combined source and sink, they may be well-suited to producing valued products without compromising photosynthesis and growth.

Combined source and sink capacities are also found in unicellular algae. Capitalizing on algal biodiversity, Treves *et al*. (2022) considered whether flux patterns of fast-growing *Chlorella ohadii* differ from those of other algae or C_3 and C_4 photosynthetic systems. *Chlorella ohadii* exhibited increased coupling of CBC to phosphoenolpyruvate that was used through anaplerosis to replenish carbon skeletons for rapid growth. The algae had a 25-fold lower 2-phosphoglycolate content than other algae, implying very low photorespiration and minimal feedback inhibition on the CBC. Observed increases in other central metabolites could offset stress or provide a stronger push of carbon through enzymes to enhance CBC flux under variable environmental conditions. The authors surmised that substrate levels may be a key to reducing the need for enzyme machinery and ensuring flexible metabolic responsiveness, which would be important for algal or crop productivity. However, flux themes may not be universally shared among photosynthetic systems because, for example, photosynthetically competent leaves are mostly exporting carbon as sucrose or producing starch, unlike algal cells, which undergo rapid cell division.

V. Current challenges and future opportunities

Isotopically nonstationary MFA offers considerable promise to advance metabolic phenotypes crucial for biotechnology and sustainability; however, realizing the potential will require: (1) improved measurements for subcellular phenomena; (2) strategies to examine unexplored cell types and organs; and (3) continued leveraging of the latest technological progress.

At the subcellular level, distinguishing compartmentalized metabolites remains challenging and methods are limited to fractionation (Arrivault *et al*., 2017) or examination of labeling in

macromolecules made from precursors with unique subcellular biosynthetic origins (Allen *et al.*, 2012). Labeled biopolymers may hold some of the keys to spatial analysis but would require adaptation to be suitable for INST-MFA. For example, amino acids in proteins translated from nuclear or plastidic genomes have distinct labeling patterns because of the differing subcellular origins of biosynthesis (Allen *et al.*, 2012); thus high-resolution-MS quantification of labeled peptides (Allen *et al.*, 2014) can provide data for peptide-based flux analysis (Mandy *et al.*, 2014). Protein amino acid sequences possess information specific to the organism (Ruhl *et al.*, 2011; Ghosh *et al.*, 2014), and tracking ectopic expression of isotope-labeled GFP can be an option to investigate phenomena specific to cell type (Rossi *et al.*, 2017). These developments provide the opportunity to match single-cell-derived fluxes with transcripts, unlabeled metabolites, and proteomic data; however, for compatibility with INST-MFA, waiting for a protein to become sufficiently labeled may not fit with time-course labeling of the metabolism of interest. Thus, challenges remain to optimize the readouts that can define spatial and, in some instances, community relationships.

Metabolism across multiple cell types, such as C_4 systems, would also benefit from enhanced spatial information. As an example, labeling kinetics in maize leaves indicate flux through phosphoenolpyruvate carboxykinase (PEPCK) is 10–25% of malic enzyme (Weissmann *et al.*, 2016; Arrivault *et al.*, 2017), though this role can be enhanced in low irradiances (Medeiros *et al.*, 2022) when inefficiencies in the carbon concentrating mechanism result in enhanced photorespiration. The authors posited that accumulation of photorespiratory intermediates may provide a reservoir for rapid consumption when CBC increases with light, which would be similar in concept to recent descriptions of photorespiratory glycine buffering in C_3 plants (Fu *et al.*, 2023). These findings suggest nonintuitive metabolic operation because they include dynamic, responsive aspects of metabolism, which are highly relevant in the field and infrequently evaluated in controlled lab settings; however, rapid changes in metabolism may compromise the application of INST-MFA, highlighting the need to further advance techniques to study dynamic metabolism more effectively.

The movement of isotopes between cells and tissues within a short time frame poses challenges in non-foliar tissues. A recent study (Smith *et al.*, 2022) highlighted persistent challenges when applying INST-MFA to heterotrophic cell systems where intercellular diffusion of supplied carbon sources may be slow and impact interpretation. Isotopic studies to assess lignin biosynthesis in stems (Guo *et al.*, 2018; Wang *et al.*, 2018) required specialized methods to deliver ^{13}C-phenylalanine for flux maps. Transient ^{13}C-analysis in reproductive tissues of sorghum identified a role for pedicellate spikelets in grain productivity (AuBuchon-Elder *et al.*, 2020), signifying the importance of non-foliar organs, but as of yet a multi-organ map exploring this synergy in sorghum does not exist. In the future, applications that widen the scope of INST-MFA to other organs or their combination may benefit from complementary advances in imaging that can quantitatively asses aspects of cells and tissues (Rolletschek *et al.*, 2021; Borisjuk *et al.*, 2023) and in some instances can resolve isotopologues (Romsdahl *et al.*, 2021).

Fundamental to comprehensive INST-MFA is the choice of isotopes and their use alone or in tandem. A historic example of the power of dual labeling capitalized on the differential turnover of lipids enriched with ^{13}C^{18}O carboxyl groups in fatty acids (Pollard & Ohlrogge, 1999) to distinguish flux through duplicated lipid assembly pathways differing by subcellular location in the endoplasmic reticulum or chloroplast. Current advances in high-resolution-MS (HRMS) can distinguish small differences in *m/z* due to differing elements isotopologues. Advances in computational tools (Borah Slater *et al.*, 2023) that can incorporate isotopes of multiple elements as separate experiments (Callaghan *et al.*, 2023), and those that utilize HRMS to distinguish isotopes from multi-element single-labeling experiments resulting in more sensitive flux assessment (Kambhampati *et al.*, 2024), represent a next frontier for INST-MFA.

VI. Conclusions

The comprehensive descriptions afforded by MFA studies have produced novel insights, not possible through other techniques. Advances in software and instrumentation in support of INST-MFA now provide a near limitless opportunity to rigorously examine most aspects of plant metabolism quantitatively. Examples presented here highlight the potential for INST-MFA to inspire efforts to augment cellular metabolism that results in increased crop productivity in lieu of less favorable climates and environmental conditions.

Acknowledgements

The authors thank Dr Stewart A. Morley (Donald Danforth Plant Science Center) for the soybean seed culturing image used in Fig. 2 and Dr Kirk J. Czymmek (Advanced Bioimaging Laboratory, Donald Danforth Plant Science Center) for the picture of the camelina silique used in Fig. 3. The authors acknowledge the USDA-ARS and Donald Danforth Plant Science Center including the Subterranean Influences on Nitrogen and Carbon Cycling (SINC) Center. In addition, this effort was supported by the Department of Energy Office of Science, Office of Biological and Environmental Research (BER) Award No. DE SC0023142 and DE-SC0022207, National Science Foundation Award No. IOS-1829365 and USDA National Institute of Food and Agriculture Grant No. 2021-67013-33778.

Competing interests

None declared.

Author contributions

SK, PJ, ML and DKA conceived of all ideas for the review and participated in writing and editing the manuscript.

ORCID

Doug K. Allen https://orcid.org/0000-0001-8599-8946
Somnath Koley https://orcid.org/0000-0002-5385-3395
Maneesh Lingwan https://orcid.org/0000-0003-2757-3012

References

Allen DK. 2016. Quantifying plant phenotypes with isotopic labeling & metabolic flux analysis. *Current Opinion in Biotechnology* **37**: 45–52.

Allen DK, Bates PD, Tjellström H. 2015. Tracking the metabolic pulse of plant lipid production with isotopic labeling and flux analyses: past, present and future. *Progress in Lipid Research* **58**: 97–120.

Allen DK, Goldford J, Gierse JK, Mandy D, Diepenbrock C, Libourel IGL. 2014. Quantification of peptide m/z distributions from ^{13}C-labeled cultures with high-resolution mass spectrometry. *Analytical Chemistry* **86**: 1894–1901.

Allen DK, Laclair RW, Ohlrogge JB, Shachar-Hill Y. 2012. Isotope labelling of Rubisco subunits provides *in vivo* information on subcellular biosynthesis and exchange of amino acids between compartments. *Plant, Cell & Environment* **35**: 1232–1244.

Allen DK, Ratcliffe RG. 2009. Quantification of isotope label. In: Schwender J, ed. *Plant metabolic networks*. Springer New York: New York, NY, USA, 105–149.

Allen DK, Young JD. 2013. Carbon and nitrogen provisions alter the metabolic flux in developing soybean embryos. *Plant Physiology* **161**: 1458–1475.

Allen DK, Young JD. 2020. Tracing metabolic flux through time and space with isotope labeling experiments. *Current Opinion in Biotechnology* **64**: 92–100.

Antoniewicz MR. 2013. ^{13}C metabolic flux analysis: optimal design of isotopic labeling experiments. *Current Opinion in Biotechnology* **24**: 1116–1121.

Arrivault S, Guenther M, Fry SC, Fuenfgeld MM, Veyel D, Mettler-Altmann T, Stitt M, Lunn JE. 2015. Synthesis and use of stable-isotope-labeled internal standards for quantification of phosphorylated metabolites by LC-MS/MS. *Analytical Chemistry* **87**: 6896–6904.

Arrivault S, Obata T, Szecowka M, Mengin V, Guenther M, Hoehne M, Fernie AR, Stitt M. 2017. Metabolite pools and carbon flow during C_4 photosynthesis in maize: $^{13}CO_2$ labeling kinetics and cell type fractionation. *Journal of Experimental Botany* **68**: 283–298.

AuBuchon-Elder T, Coneva V, Goad DM, Jenkins LM, Yu Y, Allen DK, Kellogg EA. 2020. Sterile spikelets contribute to yield in sorghum and related grasses. *Plant Cell* **32**: 3500–3518.

Borah Slater K, Beyss M, Xu Y, Barber J, Costa C, Newcombe J, Theorell A, Bailey MJ, Beste DJV, McFadden J *et al.* 2023. One-shot $^{13}C^{15}N$-metabolic flux analysis for simultaneous quantification of carbon and nitrogen flux. *Molecular Systems Biology* **19**: e11099.

Borisjuk L, Horn P, Chapman K, Jakob PM, Gundel A, Rolletschek H. 2023. Seeing plants as never before. *New Phytologist* **238**: 1775–1794.

Busch FA, Sage RF, Farquhar GD. 2018. Plants increase CO_2 uptake by assimilating nitrogen via the photorespiratory pathway. *Nature Plants* **4**: 46–54.

Callaghan MM, Thusoo E, Sharma BD, Getahun F, Stevenson DM, Maranas C, Olson DG, Lynd LR, Amador-Noguez D. 2023. Deuterated water as a substrate-agnostic isotope tracer for investigating reversibility and thermodynamics of reactions in central carbon metabolism. *Metabolic Engineering* **80**: 254–266.

Carey LM, Clark TJ, Deshpande RR, Cocuron JC, Rustad EK, Shachar-Hill Y. 2020. High flux through the oxidative pentose phosphate pathway lowers efficiency in developing Camelina seeds. *Plant Physiology* **182**: 493–506.

Chu KL, Koley S, Jenkins LM, Bailey SR, Kambhampati S, Foley K, Arp JJ, Morley SA, Czymmek KJ, Bates PD *et al.* 2022. Metabolic flux analysis of the non-transitory starch tradeoff for lipid production in mature tobacco leaves. *Metabolic Engineering* **69**: 231–248.

Clapero V, Arrivault S, Stitt M. 2023. Natural variation in metabolism of the Calvin–Benson cycle. *Seminars in Cell & Developmental Biology* **155**: 23–36.

Fernie AR, Stitt M. 2012. On the discordance of metabolomics with proteomics and transcriptomics: coping with increasing complexity in logic, chemistry, and network interactions scientific correspondence. *Plant Physiology* **158**: 1139–1145.

Fu X, Gregory LM, Weise SE, Walker BJ. 2023. Integrated flux and pool size analysis in plant central metabolism reveals unique roles of glycine and serine during photorespiration. *Nature Plants* **9**: 169–178.

Ghosh A, Nilmeier J, Weaver D, Adams PD, Keasling JD, Mukhopadhyay A, Petzold CJ, Martin HG. 2014. A peptide-based method for ^{13}C metabolic flux analysis in microbial communities. *PLoS Computational Biology* **10**: e1003827.

Guo L, Wang P, Jaini R, Dudareva N, Chapple C, Morgan JA. 2018. Dynamic modeling of subcellular phenylpropanoid metabolism in Arabidopsis lignifying cells. *Metabolic Engineering* **49**: 36–46.

Kambhampati S, Hubbard AH, Koley S, Gomez JD, Marsolais F, Evans BS, Young JD, Allen DK. 2024. SIMPEL: using stable isotopes to elucidate dynamics of context specific metabolism. *Communications Biology* **7**: 172.

Koley S, Chu KL, Gill SS, Allen DK. 2022a. An efficient LC-MS method for isomer separation and detection of sugars, phosphorylated sugars, and organic acids. *Journal of Experimental Botany* **73**: 2938–2952.

Koley S, Chu KL, Mukherjee T, Morley SA, Klebanovych A, Czymmek KJ, Allen DK. 2022b. Metabolic synergy in Camelina reproductive tissues for seed development. *Science Advances* **8**: eabo7683.

Koley S, Grafahrend-Belau E, Raorane ML, Junker BH. 2020. The mevalonate pathway contributes to monoterpene production in peppermint. *bioRxiv*. doi: 10.1101/2020.05.29.124016.

Kruger NJ, Masakapalli SK, Ratcliffe RG. 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.

Kruger NJ, Ratcliffe RG. 2015. Fluxes through plant metabolic networks: measurements, predictions, insights and challenges. *The Biochemical Journal* **465**: 27–38.

Ma FF, Jazmin LJ, Young JD, Allen DK. 2014. Isotopically nonstationary ^{13}C flux analysis of changes in *Arabidopsis thaliana* leaf metabolism due to high light acclimation. *Proceedings of the National Academy of Sciences, USA* **111**: 16967–16972.

Mandy DE, Goldford JE, Yang H, Allen DK, Libourel IG. 2014. Metabolic flux analysis using ^{13}C peptide label measurements. *The Plant Journal* **77**: 476–486.

Masakapalli SK, Bryant FM, Kruger NJ, Ratcliffe RG. 2014. The metabolic flux phenotype of heterotrophic Arabidopsis cells reveals a flexible balance between the cytosolic and plastidic contributions to carbohydrate oxidation in response to phosphate limitation. *The Plant Journal* **78**: 964–977.

Masakapalli SK, Kruger NJ, Ratcliffe RG. 2013. The metabolic flux phenotype of heterotrophic Arabidopsis cells reveals a complex response to changes in nitrogen supply. *The Plant Journal* **74**: 569–582.

Matsuda F, Maeda K, Taniguchi T, Kondo Y, Yatabe F, Okahashi N, Shimizu H. 2021. MFAPY: an open-source PYTHON package for ^{13}C-based metabolic flux analysis. *Metabolic Engineering Communications* **13**: e00177.

Medeiros DB, Ishihara H, Guenther M, Rosado de Souza L, Fernie AR, Stitt M, Arrivault S. 2022. $^{13}CO_2$ labeling kinetics in maize reveal impaired efficiency of C_4 photosynthesis under low irradiance. *Plant Physiology* **190**: 280–304.

Morley SA, Ma F, Alazem M, Frankfater C, Yi H, Burch-Smith T, Clemente TE, Veena V, Nguyen H, Allen DK. 2023. Expression of malic enzyme reveals subcellular carbon partitioning for storage reserve production in soybeans. *New Phytologist* **239**: 1834–1851.

Pollard M, Ohlrogge J. 1999. Testing models of fatty acid transfer and lipid synthesis in spinach leaf using *in vivo* oxygen-18 labeling. *Plant Physiology* **121**: 1217–1226.

Rahim M, Ragavan M, Deja S, Merritt ME, Burgess SC, Young JD. 2022. INCA 2.0: a tool for integrated, dynamic modeling of NMR- and MS-based isotopomer measurements and rigorous metabolic flux analysis. *Metabolic Engineering* **69**: 275–285.

Rolletschek H, Mayer S, Boughton B, Wagner S, Ortleb S, Kiel C, Roessner U, Borisjuk L. 2021. The metabolic environment of the developing embryo: a multidisciplinary approach on oilseed rapeseed. *Journal of Plant Physiology* **265**: 153505.

Romsdahl TB, Kambhampati S, Koley S, Yadav UP, Alonso AP, Allen DK, Chapman KD. 2021. Analyzing mass spectrometry imaging data of ^{13}C-labeled phospholipids in *Camelina sativa* and *Thlaspi arvense* (pennycress) embryos. *Metabolites* **11**: 148.

Roscher A, Kruger NJ, Ratcliffe RG. 2000. Strategies for metabolic flux analysis in plants using isotope labelling. *Journal of Biotechnology* **77**: 81–102.

Rossi MT, Kalde M, Srisakvarakul C, Kruger NJ, Ratcliffe RG. 2017. Cell-type specific metabolic flux analysis: a challenge for metabolic phenotyping and a potential solution in plants. *Metabolites* **7**: 59.

Ruhl M, Hardt WD, Sauer U. 2011. Subpopulation-specific metabolic pathway usage in mixed cultures as revealed by reporter protein-based ^{13}C analysis. *Applied and Environmental Microbiology* **77**: 1816–1821.

Schwender J. 2023. Walking the 'design-build-test-learn' cycle: flux analysis and genetic engineering reveal the pliability of plant central metabolism. *New Phytologist* **239**: 1539–1541.

Schwender J, Konig C, Klapperstuck M, Heinzel N, Munz E, Hebbelmann I, Hay JO, Denolf P, De Bodt S, Redestig H *et al.* 2014. Transcript abundance on its own cannot be used to infer fluxes in central metabolism. *Frontiers in Plant Science* **5**: 668.

Sharkey TD, Preiser AL, Weraduwage SM, Gog L. 2020. Source of ^{12}C in Calvin–Benson cycle intermediates and isoprene emitted from plant leaves fed with $^{13}CO_2$. *The Biochemical Journal* **477**: 3237–3252.

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

Smith EN, Ratcliffe RG, Kruger NJ. 2022. Isotopically non-stationary metabolic flux analysis of heterotrophic *Arabidopsis thaliana* cell cultures. *Frontiers in Plant Science* **13**: 1049559.

Stitt M, Luca Borghi G, Arrivault S. 2021. Targeted metabolite profiling as a top-down approach to uncover interspecies diversity and identify key conserved operational features in the Calvin–Benson cycle. *Journal of Experimental Botany* **72**: 5961–5986.

Szecowka M, Heise R, Tohge T, Nunes-Nesi A, Vosloh D, Huege J, Feil R, Lunn J, Nikoloski Z, Stitt M *et al.* 2013. Metabolic fluxes in an illuminated Arabidopsis rosette. *Plant Cell* **25**: 694–714.

Terashima I, Hanba YT, Tholen D, Niinemets U. 2011. Leaf functional anatomy in relation to photosynthesis. *Plant Physiology* **155**: 108–116.

Treves H, Kuken A, Arrivault S, Ishihara H, Hoppe I, Erban A, Hohne M, Moraes TA, Kopka J, Szymanski J *et al.* 2022. Carbon flux through photosynthesis and central carbon metabolism show distinct patterns between algae, C_3 and C_4 plants. *Nature Plants* **8**: 78–91.

Vanhercke T, Divi UK, El Tahchy A, Liu Q, Mitchell M, Taylor MC, Eastmond PJ, Bryant F, Mechanicos A, Blundell C *et al.* 2017. Step changes in leaf oil accumulation via iterative metabolic engineering. *Metabolic Engineering* **39**: 237–246.

Wang P, Guo L, Jaini R, Klempien A, McCoy RM, Morgan JA, Dudareva N, Chapple C. 2018. A ^{13}C isotope labeling method for the measurement of lignin metabolic flux in Arabidopsis stems. *Plant Methods* **14**: 51.

Weissmann S, Ma F, Furuyama K, Gierse J, Berg H, Shao Y, Taniguchi M, Allen DK, Brutnell TP. 2016. Interactions of C_4 subtype metabolic activities and transport in maize are revealed through the characterization of DCT2 mutants. *Plant Cell* **28**: 466–484.

Wieloch T, Augusti A, Schleucher J. 2022. Anaplerotic flux into the Calvin–Benson cycle: hydrogen isotope evidence for *in vivo* occurrence in C_3 metabolism. *New Phytologist* **234**: 405–411.

Wu C, Guarnieri M, Xiong W. 2023. FreeFlux: a Python package for time-efficient isotopically nonstationary metabolic flux analysis. *ACS Synthetic Biology* **12**: 2707–2714.

Xu Y, Fu X, Sharkey TD, Shachar-Hill Y, Walker ABJ. 2021. The metabolic origins of non-photorespiratory CO_2 release during photosynthesis: a metabolic flux analysis. *Plant Physiology* **186**: 297–314.

Xu Y, Wieloch T, Kaste JAM, Shachar-Hill Y, Sharkey TD. 2022. Reimport of carbon from cytosolic and vacuolar sugar pools into the Calvin–Benson cycle explains photosynthesis labeling anomalies. *Proceedings of the National Academy of Sciences, USA* **119**: e2121531119.

Zhang X, Misra A, Nargund S, Coleman GD, Sriram G. 2018. Concurrent isotope-assisted metabolic flux analysis and transcriptome profiling reveal responses of poplar cells to altered nitrogen and carbon supply. *The Plant Journal* **93**: 472–488.