Plant Metabolic Networks

Jörg Schwender
Editor

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Foreword by Jacqueline V. Shanks

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Foreword

I am delighted to see this pioneering edited book on plant metabolic networks, by Schwender. Now, and increasingly in the future, plant biotechnology will be key element in enabling a renewable and sustainable world. Plants and their products impact several economic sectors of society – food, feed, materials, environmental aesthetics, pharmaceuticals, fuels, and feedstocks for the chemical industry. With the advances in plant genomics, the plant researcher has a wealth of technologies available for enhanced plant productivity, but is left to ponder what the best approaches to link genotype to phenotype are. The linchpin in this link is the plant metabolic networks that govern plant product synthesis. The time is right for training many young scientists and engineers to develop and maximize the knowledge base of plant metabolic networks for the rational design of improved plant varieties.

Unfortunately, there have been relatively few complete and technical books on quantitative analysis of metabolic networks, in plants in particular. Schwender brings his experience in, and enthusiasm for, plant metabolic networks to this book. His personal research experience in flux analysis is clear in guiding the organization and content of this book, and adds a tremendous amount of practical insight and relevance. One of the most impressive aspects of this book is its broad coverage by expert and active researchers in the field of the challenges involved in analyzing plant networks. Two highly divergent approaches are covered – the "omics" view that uses global measurements of network parts (metabolites, transcripts, proteins) and statistics to deduce correlative interactions versus the mechanistic view that uses modeling and is typically not as comprehensive. This book will assist the plant researcher in deciding when to use the "omics" approach versus the mechanistic one, and how information for both approaches can be interpreted and consolidated into working hypotheses of the functioning of plant networks.

Part I sets the stage and discusses the unique complexity of plant metabolism. I am pleased to see that model organisms are described and encouraged as a general framework for describing and understanding plant networks. In addition, two important areas of active research, transport processes and metabolons, are highlighted. The compartmentation within and between cells is one of the distinctive aspects of plant metabolism, which gives plants "plasticity" to adapt to many different environments. Bottlenecks in plant metabolic engineering can often be tied to these two topics. Regarding transport, having the metabolite in the right place at the right time

to undergo catalysis may be a rate-limiting step in making the desired product. For metabolons, multienzyme complexes of several enzymes, overexpression of one enzyme in the complex may not affect the overall rate. These complexes will have to be modeled differently than single enzymes – the enzymes will be tightly coupled and coordinated. Hopefully, the material in these chapters will help prevent "irrational" engineering by future plant biotechnologists!

Part II examines the key measuring parts of the metabolic network: i.e., metabolites and enzyme kinetics. The technologies and associated challenges to measure both of these quantities are straightforwardly described. With the tremendous amount and variety of chemistries in the plant cell, and with the number of methodologies for quantification of metabolites, including ^{13}C-labeled ones, this is no small feat! Here, the plant researcher can deduce major strategic decisions that they must grapple with – how to balance breadth versus depth, both in terms of the measurement quality and the statistical analysis required to analyze the information. These questions are ones that must be continuously examined by the researcher in any organization.

Part III discusses the analytical and organizational challenges that all researchers of plant metabolic networks must tackle: how to analyze, interpret, and/or model the data in order to draw relevant conclusions. Virtually, every technologist inevitably wonders whether they need modeling. This is especially true, given the varying mathematical backgrounds of the researchers – leading to a significant fraction with an inherent distrust of models. This section debunks the mystique of the modeling process, to enable a larger audience to comfortably understand the huge potential and limitations of these vital tools for analyzing metabolic networks. Understanding of the model structure, assumptions in building the model, and the uncertainty in data and model parameters will enable the researcher to critically assess the model results, resulting in increased power to generate testable hypotheses that by inspection alone one cannot muster. After reading these chapters, the researcher will become more comfortable with the uncertainty inherent in metabolism.

The quantitative analysis of plant metabolic networks is intrinsically interdisciplinary in nature – hence the challenges in training and utilization of these technologies. In looking through this practical and comprehensive treatment, my first reaction was, "I wish I had available a book like this when I had begun to mentor my students, before they started analyzing plant networks!" A similar reaction from my students might have been, "I wish I had read a book like this when I began my research project so that I knew what she was talking about!" Unfortunately, we had to learn many of the topics covered here in real time.

This book does justice to the current state of the art for analyzing plant metabolic networks. In my experience, the challenges discussed in this book have really proved to be the hurdles to overcome.

With this new book, a new generation will get to share the extensive and deep insights of Schwender and the books' contributors, push the frontiers in quantitative plant network analysis, and become tomorrow's industrial and academic leaders in plant biotechnology.

Ames, Iowa Jacqueline V. Shanks

Preface

The main motivation behind *Plant Metabolic Networks* was to bring together in one book various, and in part, very diverse approaches that relate to the quantitative analysis of metabolic networks in plants. Although flux analysis and related approaches are not really new, their recent re-emergence in plant science made it worthwhile to summarize recent advances in this area, and to give an overview of the current state of knowledge. Expert authors from leading groups in plant science could be convinced to contribute the 11 chapters to the book. I am very thankful to all the contributors for their time and effort spent in writing the chapters, and I hope that this book might be useful for plant biologists, students, and researchers in related fields in order to stimulate further progress in plant metabolic research and biotechnology.

Plant Metabolic Networks is intended to be in part tutorial, and in part review of recent literature, and hopefully turns out to be more than a collection of review articles. Two basic areas are touched: experimental/analytical techniques needed to produce metabolic data, and the mathematical modeling used to analyze these data. The authors kept the mathematical detail at a minimum to make the book accessible for a broad audience of plant biologists. However, the metabolic research described in this book certainly is of interdisciplinary nature and mathematicians who are interested in analyzing plant metabolic networks should benefit from the book as well.

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Chapter 1
Introduction

Jörg Schwender

1.1 Why a Book on Plant Metabolic Networks?

With several complete plant genomes currently available and with massive amounts of transcriptomic, proteomic, and metabolomic data being generated by plant scientists, one could assume that making sense of all this data is only a minor problem. However, it has been pointed out by many leading plant scientists that this is not the case and that developing appropriate modeling skills and tools may lack behind the technological progress that allows the data generation. In recent years, the plant science community became more and more aware of the importance of different kinds of analysis and modeling approaches, like metabolic flux analysis. Accordingly, in this book, contributions from different expert authors have been assembled to give a current view on plant metabolic networks, from the analysis of the molecular parts to approaches of mathematical modeling of plant metabolic networks at the cellular level. Other processes like gene regulation, cell signaling or models at the whole plant or ecosystem level certainly have their justification [1], but have been mostly excluded here to give cellular metabolism special attention.

1.1.1 What Constitutes Metabolism and Metabolic Networks?

Metabolism can be defined as a highly organized, self-regulated, and continuous process of uptake, transport, chemical modification, and secretion of small molecules. As pointed out by Wiechert et al. [2], metabolism has its manifestation in two quantities: concentration and flux – with analogy to potential and flow in many different physical systems. Flow is causal for potential and vice versa [2]. This means that if the momentary metabolic state of a cell is to be described, both of them have to be determined.

The many specific interactions between the multitude of different metabolites and enzymes constitute a complex network. There is increasing awareness among plant

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scientists that analyzing the systems properties of these networks and the use of predictive models of metabolism will be of major importance for further progress in understanding of plants at the molecular level [3]. Systems properties can describe a system at its whole but are destroyed if the system is dissected into its elements. Systems theory assumes that there are different levels of organization with additional properties arising at each level ("emerging properties") [4]. Metabolic networks are one level at which properties like elementary flux modes, metabolic flux, centrality or robustness emerge.

1.1.2 Why Study Metabolic Networks?

In difference to protein–protein or protein–nucleic acid interaction networks, metabolic networks, in particular, can be modeled in a very exact way. For example, the stoichiometric coefficients in the mass balance of an enzyme-catalyzed reaction are exact integer numbers. This may in part explain the success of the different modeling approaches described and discussed in this book.

In an attempt to categorize current approaches to model plant metabolism, two groups of approaches can be discerned. Global inquiry and discovery (Chapters 3 and 6) is based on global measurement of network compounds (metabolites, transcripts, proteins), typically in response to different kinds of perturbation (e.g., growth condition, transgenic events). Mainly by use of multivariate statistics, this approach typically identifies components that co-respond to a stimulus and therefore should have close functional relationship. In fact, it has been found that the metabolome reacts in a very sensitive and dynamic way to many kinds of perturbation (see Chapter 6). In this way, e.g., metabolites belonging to a metabolic pathway can be identified. This approach can be applied to diverse plant systems and most often captures the effect of perturbation globally. It also can integrate across the organizational levels (metabolome, transcriptome, see Chapter 6). However, the discovered underlying correlative relationship has subsequently to be investigated in detail to reveal the molecular mechanisms behind.

An apparently diametrical approach tries to build mechanistic models based on functions already known with some detail, such as reaction stoichiometry or enzyme mechanisms. Systems properties on the level of metabolism can be analyzed and predicted by these models and then verified on basis of experiments. This approach receives increasing interest in the plant science community during recent years as documented in several chapters of this book. Due to the apparent complexity of metabolism, compromise in the level of detail represented in mechanistic models is unavoidable, i.e., by far not all cellular reactions may be represented. Also, models bridging several organizational levels are still in their infancy, i.e., metabolic models often ignore genetic regulation or the dynamics of the metabolic network during development. Therefore one has always to be aware of the limited validity of this kind of modeling. In addition, the experimental component of this approach can be quite demanding and not easily applicable to whole plants. For example, if

metabolic flux is to be measured, issues like metabolic non-steady state or heterogeneous cell types in tissues may result in misleading results.

For many plant scientist, mathematical and computational procedures are a hurdle in applying mechanistic models to plant metabolism – and hopefully this book is helpful to alleviate this problem.

1.2 Contents of the Book

The book can be divided into three parts: First an introductory chapter (Chapter 2), relating to the unique complexity of plant metabolism. The following three chapters describe how to analyze the components that make up the metabolic network, metabolites, and enzymes. Finally, Chapters 6–11 are devoted to network analysis and modeling.

1.2.1 Complexity of Plant Metabolism

Chapter 2 introduces the elucidation of metabolic pathways in plants, pointing out the importance of model organisms. While plants are characterized by a complex organization into different cell types as well as by subcellular compartments with different metabolic activity, big parts of core plant metabolic pathways had been revealed by using simple model organisms such as unicellular green algae. The choice of such a uniform cell type, as an experimental system, was critical to elucidate the structure of key metabolic routes in sufficient detail. Therefore, the author encourages plant scientists to keep using some of these model organisms in research on plant metabolism.

The discovery of pathway/network structure in plants is far from being completed. Due to the complex organization of plant metabolism into different cell types and subcellular compartments, transport processes and transport proteins are critical parts of plant metabolism and by far not all of them have been identified and characterized yet. For some metabolic pathways, the particular steps are distributed across several subcellular compartments and even across different cell types. Here, highly selective metabolite transporters take part in the control of metabolism and cross-talk between compartments. For example, the photorespiratory cycle and C4 photosynthesis are both highly compartmentalized pathways and have been long described in textbooks. However, the transport between the organelles is not yet understood in detail at the molecular level, and many of the transporters involved have yet to be identified.

Chapter 2 goes on to show that another important but not well-studied issue in the organization of plant metabolism is the association of enzymes in metabolons. Metabolons are macromolecular complexes of enzymes in which metabolic intermediates are passed on from the active site of one enzyme to the next. Metabolon organization has been detected in plant glycolysis, in photorespiration, but is also

supposed to be present in Calvin cycle and secondary metabolism [5]. Recognizing the importance of macromolecular associations of enzymes, it is clear that more structural studies in enzyme proteins and protein complexes will be necessary.

The definition of metabolic networks is possible based on genomic information. With lower cost and faster throughput in sequencing technology, the number of plant genomes is rapidly increasing. The genome information basically contains all the genes that make up the metabolic network. For the reconstruction of genome scale metabolic networks, it is important to note that such methods do not permit the assignment of functions to unknown genes. Here, the author predicts that that integration of different omics data types will be helpful. The author predicts that integration of multivariate datasets from different "omics" approaches might eventually permit the ab initio computational deduction of complex networks.

1.2.2 Measurement of Network Components

The second part of the book is devoted to the generation of data that quantify and describe the components of metabolic networks – metabolites and the enzymes.

Chapter 3 gives an overview over experimental approaches and technologies that can be used to measure, identify, and quantify the vast amount of metabolites found in plants. While these technologies are constantly evolving, GC/MS with electron impact ionization is probably still the mostly used technology in metabolic profiling and metabolomics, due to instrumentation cost, well-established methods, and readily available mass spectral libraries. To just mention recent technologies not reviewed here: Mass spectrometric imaging approaches (see e.g., Zhang et al. [6]) may represent an important progress in the field. While classical metabolite profiling always uses extraction and separation/analysis of whole tissues (global analysis), these more recent developments may be key to resolve the metabolism of the different cell types.

Chapter 4 introduces the kinetic properties of enzymes and the parameters that describe them and are needed to model metabolic networks dynamically. The chapter then goes on to put emphasis on recent advances in high throughput profiling of total enzyme activities as well as of apparent kinetic constants in extracts of plant tissues. The enzyme analysis platform described here approaches the high throughput scale, which is very desirable and already routine in metabolomics, transcriptomics, or proteomics.

In Chapter 5, the analysis of metabolite labeling in stable isotope labeling experiments is reviewed in much detail. The resulting data are basis for ^{13}C-based metabolic flux analysis. Isotope tracers can be used to follow the metabolic fate of specific atoms through the metabolic network and to determine mass flows. First, procedures commonly used in plant flux analysis for extraction of plant tissue and subsequent fractionation and derivatization of the labeled compounds are described. Quantification of stable isotope label by nuclear magnetic resonance (NMR) is reviewed with respect to one-dimensional and two-dimensional NMR techniques that have been applied. In NMR, fractional enrichment (i.e., position-specific

isotope enrichment) as well as isotopomer information can be obtained. Then the often used analysis of label by gas chromatography/mass spectrometry (GC/MS) is discussed. MS has the advantage of high sensitivity and accuracy and is in particular powerful if different fragments of one molecule can be analyzed. Different aspects of chromatographic separation, ionization, ion fragmentation, and detector properties are discussed, which are the basis for accurate quantification of isotope label. Finally, the correction of mass spectrometric isotopomer data for the occurrence of natural isotopes is laid out in detail.

1.2.3 Network Modeling and Analysis

The third part of the book is devoted to the analysis of metabolic networks with different approaches from topological analysis via stoichiometric models to the dynamic simulation of biochemical reactions.

The metabolome, the complete set of small-molecule metabolites, changes its quantitative composition in response to different perturbations and stimuli. Metabolomic data indicate functional relations between metabolites. Accordingly, the processing and analysis of metabolomic data is reviewed in Chapter 6, where also the challenge of integration of different "omics" data is highlighted.

In order to describe topological features of metabolic networks, graph-theoretical mathematical approaches are reviewed in Chapter 7. Different properties like network motifs and diverse centrality measures help to understand network structures and to compare networks. The chapter also introduces various visualization tools that can be used to analyze complex data generated by metabolite profiling or transcriptomics. This includes mapping of such data on network structures. Specific reference is given to dynamic visualization of data.

The subsequent three chapters are all related to constraint-based steady-state flux analysis, which is based on the stoichiometries of biochemical reactions and on reaction directionality/reversibility. In Chapter 8, stoichiometry-based models are discussed, which allow to analyze and predict the theoretical flux capabilities of metabolic networks. The chapter first introduces the fundamentals of stoichiometric modeling. Then flux balance analysis (FBA) is introduced, which allows to predict the usage of pathways under the assumption of a certain cellular objective, such as maximal yield in biomass compounds. Different variants of flux balance analysis like minimization of metabolic adjustment (MOMA) and dynamic FBA are well explained. While these approaches are based on reaction stoichiometry, some information on reaction directionality has to be known as well. This and other thermodynamic considerations that are current limitations to the reliability of the standard FBA approach are given broad space in the chapter. Following this, extreme pathway analysis and elementary mode analysis are introduced, and applications to plant systems discussed. Finally, a section on genome scale models describes model reconstruction based on genomic data. Besides the general problems of incomplete genome annotation, gaps in pathways and dead-end metabolites require manual refinement of the models. A problem specific to eukaryotic cells is the presence of

subcellular compartments. This means that for each enzyme, the subcellular localization has to be known as well as the transport proteins that connect the compartments. In addition, to realistically simulate plants, the stoichiometry-based approach has to be extended to specific cell types.

Chapter 9 is focused on the estimation of intracellular flux, based on stable isotope-labeling experiments. The chapter relates in particular to the steady-state labeling approach, i.e., interpretation of labeling pattern in metabolites after the distribution of isotope label has reached steady state. First, application of the methodology to plants and main insights gained from such studies on plant metabolism are reviewed. Then the basic experimental setup of a steady-state labeling experiment is described and important assumptions inherent to the experimental approach, such as the approximation of metabolic and isotopic steady state, are discussed. Some characteristic features of metabolic networks used in ^{13}C-flux analysis are highlighted. Different approaches for the interpretation of labeling data and estimation of fluxes are reviewed with detailed consideration given to the underlying basic relation between labeling signatures and flux. Computational aspects of flux analysis are discussed with regards to the limits to the complexity of networks that can be analyzed, as well as related to the reliability of flux values obtained by flux-parameter fitting. Finally, the software tools available for ^{13}C-MFA are discussed.

In addition to the steady-state isotope labeling described in Chapter 9, Chapter 10 discusses the study of plant metabolic networks by dynamic flux analysis. The mathematical formalism is introduced, and classical examples of dynamic labeling as well as most recent studies on secondary plant metabolism are discussed. Dynamic flux analysis is built on the principle of the classical pulse-chase experiment. After feeding a labeled precursor, labeling pattern in metabolites are monitored in a time course. In difference to steady-state labeling, multiple samples of the time course have to be analyzed, as well as intracellular metabolite concentrations have to be measured. However, as discussed in detail in this chapter, dynamic labeling allows to circumvent some of the shortcomings of steady-state flux analysis. Actually from the famous pioneer work of Calvin Bassham and Benson until today, dynamic labeling has been proved to be a powerful tool in pathway discovery in plants. Careful interpretation of labeling time courses can establish precursor–product relationships.

Chapter 11 gives a basic introduction into kinetic modeling of plant metabolic networks. Based on a literature example, the setup, simulation, validation, and use of a kinetic model is explained. Also, metabolic control analysis (MCA) is addressed as many studies use this approach of sensitivity analysis to characterize kinetic models. This includes experimental MCA, which has regularly been applied to plants.

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Chapter 2
Definition of Plant Metabolic Networks

Andreas P.M. Weber

2.1 Plant Metabolic Networks – More Complex than Anything Else?

To the best of the author's knowledge, the answer to this question is yes. In part, the enormous complexity of plant metabolic networks is due to the high degree of compartmentation of plant cells. That is, plant cells, in contrast to other non-plant eukaryotic cells, display a higher degree of compartmentation, with the chloroplast being the most prominent compartment that is exclusive to plants. In addition to having a higher degree of compartmentation, most higher plants are sessile and thus cannot escape from biotic or abiotic stressors, which promoted the evolution of a host of metabolic adaptations to the sessile lifestyle, including an impressive range of secondary metabolites that are synthesized by plants to serve as defense compounds against pathogen attack, as attractants for pollinators, and as protective agents against abiotic stress.

In this chapter, I will discuss the impact of compartmentation of plant cells on plant metabolic networks. Particular emphasis will be given to the use of unicellular model systems for deciphering metabolic routes, to solute transport across intracellular membranes, to the concept of metabolons and substrate channeling, and to the role of chloroplasts in the plant metabolic network. The role of chloroplasts will also be considered in the context of their evolution from cyanobacterial ancestors through the process of endosymbiosis because this process is a prime example for integration of two separate metabolic entities into one "super-organism" (i.e., the first plant cell).

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2.2 Keeping It Simple – Why Unicells Are Cool

From above said, it becomes clear that gaining a deeper understanding of the metabolic networks in plant cells is not a trivial pursuit. The high degree of compartmentation, the uncertainty about which metabolites are transported across organellar membranes, and a lack of knowledge about the kinetic constants of many metabolite transporters and of subcellular metabolite concentrations hamper progress. In photosynthetic tissues, rapid randomization of applied label hinders the application of metabolic flux analysis that has been applied very successfully to eukaryotic and prokaryotic unicells and in heterotrophic plant tissues, such as developing oil seeds. As described in more detail later in this chapter, further obstacle comes from the fact that, e.g., leaf tissue of land plants consists of multiple cells types, such as the epidermis, vasculature, and photosynthetic mesophyll tissue, which is frequently multilayered and differentiated into spongy and palisade parenchyma cells. Hence, tissue extracts always represent a mixture of metabolites and enzymes derived not only from various cellular compartments but also from a variety of tissues, with very different metabolic and enzymatic capacities. Some of these issues can be addressed by subcellular fractionation techniques, such as the non-aqueous fractionation of plant tissues, which provides insights into subcellular metabolite concentrations, although it does not distinguish between the individual cell types that are present in a typical leaf of a land plant. Biological imaging techniques, based on NMR, do provide some information at cellular and subcellular level, but they are restricted to relatively few metabolites, minerals, and water [84, 85, 139]. Laser-micro-dissection of fixed tissues followed by microanalytic methods such as the analysis of secondary metabolites in individual stone cells of Norway spruce [92] and single-cell sampling techniques such as measurements of sugar concentrations of individual cells of castor bean hypocotyls [154] or amino acid analysis in leaf mesophyll cells (MC) [5] is also providing additional insights.

Many of the abovementioned limitations do not apply to unicellular photosynthetic organisms, such as algae, and the "compartmentation issue" can be addressed by investigation of prokaryotic photosynthetic organisms, such as cyanobacteria which are evolutionary connected to plastids and thus might serve as models for plastid metabolism. A prime example for the dissection of a relatively complex metabolic pathway in photosynthetic organisms is the reductive pentose phosphate pathway, also known as the Calvin cycle. In series of milestone papers in the late 1940s and early 1950s, using unicellular algae such as *Scenedesmus* and *Chlorella*, as well as land plant leaves, the path of carbon in photosynthesis was elucidated. In a landmark paper, it was shown that the first labeled organic carbon compound found in *Scenedesmus* cells that were allowed to photosynthesize in the presence of labeled carbon dioxide for five seconds was phosphoglyceric acid and that the first free carbohydrate to appear was sucrose [25]. To cite from the introduction of this classical paper, "The ideal design of an experiment to determine the chemical path of carbon from carbon dioxide to the variety of plant constituents is relatively simple and straightforward. It would consist of feeding a photosynthesizing organism radioactive carbon dioxide for various lengths of time and stopping the reaction

by killing the plant. By determining those compounds into which the radioactive carbon has been incorporated for each period of illumination and, further, by determining the distribution of radioactivity within each compound, these data could then be used to construct a family of curves depicting the increase in radioactivity in each compound (and in each carbon atom of each compound) as a function of time. From a complete set of such curves it should be possible to draw a map of the path of carbon as it flows into the plant in the form of carbon dioxide and distributes itself among all the plant constituents." In the opinion of this author, there is no better way of explaining the principle, hence the quotation. This paper goes on to suggest that hexose phosphates (i.e., glucose 1-phosphate and fructose 6-phosphate), not free glucose and fructose, are the precursors of sucrose and that sucrose phosphate might be an intermediate of sucrose biosynthesis. From quantitative analysis of the amount and the kinetics of radiolabel incorporated into the hexose phosphate pool and in the glucose and fructose moieties of sucrose, they estimated the size and turnover of the hexose phosphate pool. In a later publication, the authors also show that the sugar nucleotide UDP-glucose is likely involved in biosynthesis of sucrose [24]. A key benefit of using unicellular algae for these studies was that it was possible to very rapidly stop metabolism after applying the label, thus allowing very rapid kinetic studies of metabolism. Since the algae used in these experiments were kept as cell suspension, label could be injected into the solution and the cells be killed shortly after by dumping the suspension into boiling ethanol. Similar studies would have been very difficult, if not impossible with leaf tissue of land plants. However, other metabolic pathways in plant cells, such as sucrose biosynthesis from oil via the glyoxylate cycle and gluconeogenesis [27, 86, 87], was unraveled by feeding of radioactive tracers to land plant tissues, such as castor bean embryos. Unicellular algae have been used in numerous studies of plant metabolism, and in many cases, metabolic pathways were first worked out using algae as model systems. A specific example is the discovery of the biosynthesis of isoprenoids in plants such as carotenoids, sterols and the prenyl-side chains of chlorophyll and plastochinone by the non-mevalonate pathway [131]. This study took advantage of the fact that *Scenedesmus obliquus* cells can be grown heterotrophically on ^{13}C-labeled glucose or acetate, thus permitting the application of metabolic flux analysis, based on isotopomer distribution obtained by ^{13}C-NMR spectroscopy.

Not only eukaryotic photosynthetic systems have been used to decipher metabolic pathways but also prokaryotic photosynthetic organisms have been used as model systems. For example, the process of nitrogen fixation was for the first time directly demonstrated to be localized in the heterocysts of the N-fixing cyanobacterium *Anabaena cylindrica*, using radioactive ^{13}N that was generated by proton bombardment of ^{13}C powder in combination with micro-autoradiography [174]. This study also permitted some insights into kinetics and flux of fixed nitrogen out of the heterocysts along the filament. In two follow-up papers, the same group used ^{13}N-short-term labeling (1–120s) and pulse-chase experiments to unravel the pathway of nitrogen metabolism after fixation [146, 175]. It was clearly shown that the reaction sequence moved from production of ammonia from nitrogen gas to the formation of glutamine and eventually glutamate. From simultaneous application of

specific enzyme inhibitors, it was concluded that fixed nitrogen is metabolized by glutamine synthetase/glutamate synthase pathway [175]. Photosynthetic prokaryotic unicells, such as *Synechocystis* sp. PCC 6803, are currently being rediscovered as model systems to study metabolic fluxes [133, 132]. Cyanobacteria, such as *Synechocystis*, offer the advantage of having only one metabolically active compartment, while the carbon fixation pathway is generally similar to that of higher plants. The genome of *Synechocystis* is fully sequenced, thus permitting the reconstruction of its metabolic network from its genomic sequence. Unfortunately, steady-state metabolic flux analysis is not directly applicable to autotrophic photosynthetic organisms because incorporation of labeled carbon in the form of $^{13}C–CO_2$ will eventually lead to uniform labeling of all carbon atoms, thus preventing the deduction of flux information from isotopomer analysis. However, transient metabolic flux analysis, basically following the ideas put forward by Benson and Calvin (see above), in combination with modern analytical techniques, reconstructed stoichiometric metabolic networks from genomic data, and mathematical modeling does allow the deduction of fluxes in photoautotrophic organisms [133].

2.3 Connections Are Everything – Solute Transport and Metabolic Networks

A fundamental property of metabolic networks in plants is the selective partitioning of organic metabolites among different organelles, cells, tissues, and organs. This requires various transport mechanisms to accommodate the directional transport of metabolites. Transporters participate in basic metabolism by partitioning metabolites within and between cells, and they are essential for intermediate and long-distance transport between tissues and organs, respectively. Plants assimilate inorganic carbon and nitrogen into organic compounds required for plant growth; a very large variety of metabolites are produced, and the anabolic and catabolic pathways that they feed into are complex and interconnected. Metabolic pathways are frequently partitioned between organelles, cells, or even tissues and organs. Thus, intracellular and long-distance transport processes are critical for sustaining biosynthesis, catabolism, and growth. Since transport processes potentially affect the availability of substrates or products, they also represent critical sites at which metabolism and growth can be regulated. Hence, transport processes in plants, in particular the location and kinetic properties of transporters, are essential components of metabolic networks since they frequently influence metabolic fluxes, as well as partitioning of nutrients between growth and storage.

The high degree of compartmentation of plant cells and the distribution of many metabolic pathways across several cellular compartments and, in some cases, even different cell types requires massive flux of metabolic intermediates across cellular and organellar membranes. Since most small molecules in plant cells are not membrane permeable, metabolite transporters are required to catalyze the transport of metabolites across membranes. The compartmentation of metabolic pathways

provides additional options for regulation, permits the simultaneous operation of pathways that compete for the same substrates within the same cell, and they help avoiding futile cycles. Metabolite transporters thus play critical roles in connecting the parallel and interdependent biosynthetic and catabolic pathways and thus represent the integrating elements in these metabolic networks, similar to interchanges in road networks. In vascular plants, long-distance transport is critical for the allocation of organic carbon and nitrogen compounds from their sites of synthesis to developing or reproductive plant organs that rely on import of the organic compounds for growth and development. Obviously, a plethora of multicompartment pathways and long-distance transport processes could be reviewed here to illustrate the principles; however, due to space constraints, only two specific examples, the photorespiratory C_2 oxidation cycle and the biochemical CO_2 pump of C_4 photosynthesis, will be given.

2.3.1 The Photorespiratory C_2 Oxidation Cycle – A Highly Compartmentalized and Interconnected Metabolic Route

The photosynthetic carbon-assimilating enzyme ribulose bisphosphate carboxylase-oxygenase (Rubisco) is a bifunctional enzyme. That is, it catalyzes both the productive carboxylation and non-productive oxygenation of ribulose 1,5-bisphosphate (RuBP) [20, 21, 111]. Oxygenation of RuBP leads to the production of one molecule of 3-phosphoglycerate (3-PGA) and one molecule of the toxic intermediate 2-phosphoglycolate (2-PG), which enters the photorespiratory carbon cycle (Fig. 2.1).

The detoxification of 2-PG and its recycling to 3-PGA occurs by the complex photosynthetic carbon oxidation cycle [125, 150]. Because this pathway leads to the consumption of oxygen (oxygenation of RuBP) and production of carbon dioxide (during the recycling of 2-PG) in the light, it is also called photorespiration. The specificity factor of the bifunctional enzyme Rubisco for the carboxylation reaction versus the oxygenation reaction is in the range of 80–100 for most land plants [137].

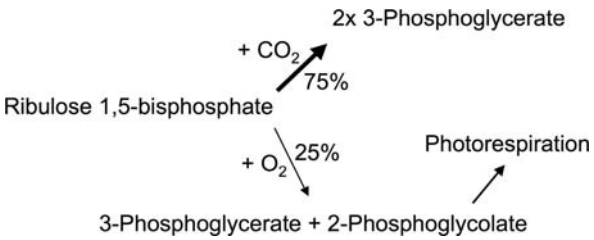

Fig. 2.1 Oxygenation reaction (+ O_2) and carboxylation (+ CO_2) reactions of Rubisco. A ratio of carboxylation to oxygenation reaction of Rubisco of 75:25 is commonly observed in C_3-type land plants

Under current atmospheric conditions, this leads to a carboxylation-to-oxygenation ratio of approximately 3:1. Hence, in C_3 plants, the rate of photorespiration would be 25% of the rate of gross CO_2 assimilation. These rates have been confirmed by short-term labeling studies of the intermediates glycolate, glycine, and serine with $^{18}O_2$ [38], by using $^{13}CO_2$ and mass spectrometry to determine CO_2 fluxes under conditions of steady-state photosynthesis [62], and from the post-illumination photorespiratory CO_2 burst [89].

2-Phosphoglycolate, one of the products of the oxygenation reaction of Rubsico, cannot be further metabolized inside the chloroplast stroma but must undergo a complex and highly compartmentalized reaction pathway in which two molecules of 2-PG are converted into one molecule of 3-PGA. This pathway minimizes the loss of fixed CO_2 and prevents the depletion of intermediates from the Calvin cycle, since 75% of the carbon of 2-PG is recycled in the photorespiratory pathway to yield 3-PGA, while 25% of the carbon is released in the form of CO_2 (Fig. 2.2).

The photorespiratory pathway represents a coordinated network consisting of 14 soluble enzymes that have been compartimentalized between chloroplasts, leaf peroxisomes, mitochondria, and cytoplasm and at least 12 transmembrane transport steps that connect the compartments (Fig. 2.3).

The photorespiratory carbon cycle is initiated in the chloroplast stroma by dephosphorylation of 2-phosphoglycolate, a reaction that is catalyzed by phosphoglycolate phosphatase (PGP). The resulting glycolate leaves the chloroplast by a glycolate/glycerate antiporter [69, 70, 179] and is taken up into the peroxisomes by an unknown transporter. Inside the peroxisomal matrix, glycolate is oxidized to glyoxylate by FMN-dependent glycolate oxidase, which catalyzes the transfer of two electrons from glycolate to O_2, yielding hydrogen peroxide (H_2O_2). Glyoxylate is transaminated to glycine by two aminotransferases – serine:glyoxylate and glutamate:glyoxylate aminotransferase (SGT and GGT, respectively [73, 93, 122]. Glycine then leaves the peroxisomes by an unknown transporter and is taken up into the mitochondria by a glycine/serine transporter [37, 36, 180]. In the mitochondrial matrix, two molecules of glycine are converted to CO_2, NH_3, NADH, and serine by the concerted actions of glycine dehydrogenase [114] and serine hydroxymethyl transferase [156]. Serine leaves the mitochondria, likely by the same transporter that also catalyzes the import of glycine, and is taken up into the peroxisomes, where the amino group of serine is removed by SGT, yielding hydroxypyruvate which is subsequently reduced to glycerate in a reaction consuming NADH by

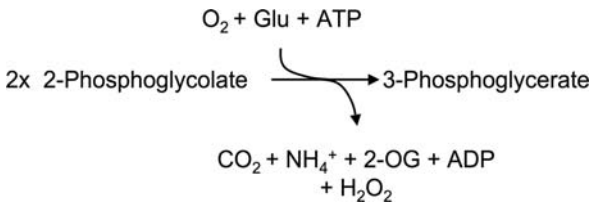

Fig. 2.2 Summary view of the photorespiratory carbon oxidation cycle

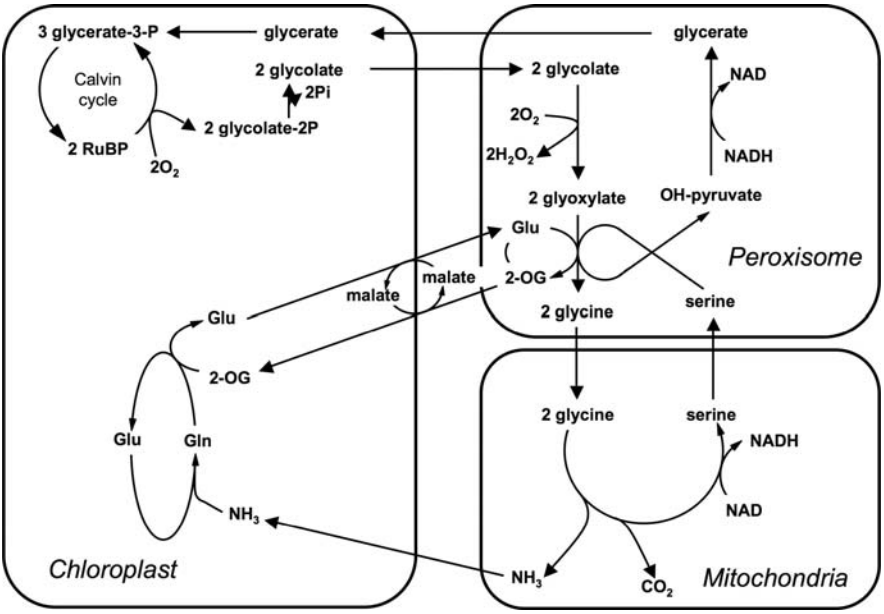

Fig. 2.3 Schematic representation of the photorespiratory carbon oxidation cycle. Please note that the redox shuttles connecting the NADH/NAD pools of mitochondria, cytoplasm, and peroxisomes via the respective isoforms of malate dehydrogenase are not shown for the sake of clarity

hydroxypyruvate reductase (HPR) [151, 181]. It has to be noted, though, that an alternative (NADPH-dependent) pathway for the reduction of hydroxypyruvate to glycerate apparently exists in the cytoplasm [81, 82]. Also, barley mutants deficient in peroxisomal HPR [107] do not display the characteristic conditional lethal phenotype at ambient CO_2 displayed by mutants deficient in other enzymes of the photorespiratory pathway [125], thus supporting the notion that a bypass exists for the reaction catalyzed by peroxisomal NADH-HPR. Glycerate, resulting from the reduction of hydroxypyruvate, is taken up into the chloroplast stroma by above-mentioned glycolate/glycerate transporter, where it is phosphorylated by stromal glycerate kinase in an ATP-dependent reaction to yield 3-PGA [14]. 3-PGA enters the Calvin cycle, thus completing the photorespiratory carbon cycle.

Although photorespiration represents one the major carbon fluxes in photosynthetic tissues of C_3 plants, many aspects related to this pathway, such as its regulation and its impact on the interaction between carbon and nitrogen metabolism are not understood [94, 160, 165]. Particularly scarce is our knowledge about the transport of photorespiratory intermediates across the membrane(s) of the three participating organelles. To date, only two metabolite transporters involved in photorespiration have been identified at the molecular level; i.e., the plastidic 2-oxoglutarate/malate and glutamate/malate translocators (DiT1 and DiT2, respectively) [124, 130, 160, 161].

2.3.2 The Biochemical CO_2 Pump of C_4 Photosynthesis – Share of Labor Between Two Cell Types Causing Massive Flux of Metabolic Intermediates

A dramatic rise of atmospheric oxygen levels and a concomitant decline of the CO_2 levels, approximately 300 million years ago, as a consequence of oxygenic photosynthesis and (bio-) geochemical processes [9, 10, 34, 127], increased the selective pressure on photosynthetic organisms to evolve carbon-concentrating mechanisms (CCM), in order to minimize the oxygenase activity of Rubisco and to thus decrease the rate of photorespiration. Whereas many cyanobacteria and algae have evolved highly efficient single-cell carbon-concentrating mechanisms [7, 147], some terrestrial plants and few aquatic plants have evolved the process of C_4 photosynthesis [44, 58, 60, 61, 75, 120]. Because the carbon-concentrating mechanism of C_4 plants alleviates the CO_2 limitation of photosynthesis that occurs in C_3 plants at high temperature due to the relative acceleration of the oxygenation reaction with increasing temperature, C_4 photosynthesis is more efficient in warmer climates or under conditions of drought and salinity. In addition to a higher carbon-use efficiency, many C_4-type plants also have higher nitrogen use efficiency than C_3-type plants [110].

Since the discovery of C_4 photosynthesis in the 1960s, much has been learned about the physiological, biochemical, and anatomical features associated with this mode of photosynthesis. Until recently, it was believed that a distinct anatomical feature, called the Kranz anatomy, is required for C_4 photosynthesis. Kranz is the German word for wreath – a "wreath" of chloroplast-containing mesophyll cells surrounds a layer of chloroplast-containing bundle sheath cells (BSC) that in turn form a second "wreath" around the vascular bundle. The recent discovery of single-cell C_4 photosynthesis in several *Chenopodiaceae* species demonstrated that Kranz anatomy is not essential for C_4 photosynthesis [43, 157, 158]. The unifying principle of single-cell and dual-cell (i.e., Kranz anatomy) C_4 photosynthesis is compartmentation – either within a single cell or between two specialized cell types. In the following, we will focus on dual-cell C_4 plants.

Common to all C_4 (and CAM) plants is that CO_2 (in the form of HCO_3^-) is initially fixed by PEP carboxylase (PEPC):

$$\mathrm{PEP} + \mathrm{HCO_3^-} \rightarrow \mathrm{OAA} + \mathrm{Pi}$$

This reaction happens in the cytosol of the mesophyll cells. The further fate of OAA differs between the three biochemical variants ($NADP^+$-malic enzyme type, NAD^+-malic enzyme type, PEP carboxykinase type) of the C_4 pathway. In this review, we will focus on the $NADP^+$-malic enzyme type plant maize; the other two variants will not be discussed.

In maize, OAA is taken up into mesophyll cell chloroplasts, reduced to malate by $NADP^+$-malate dehydrogenase (MDH); malate is subsequently transported back to the mesophyll cell cytosol. Malate is then transported to the bundle sheath cell chloroplasts, where it is decarboxylated and oxidized, yielding pyruvate, NADPH,

and CO_2. The liberated CO_2 is refixed by Rubisco. The decarboxylation product, pyruvate, is transported back to the mesophyll cells, taken up into mesophyll cell chloroplasts, and phosphorylated to regenerate the CO_2 acceptor PEP by pyruvate phosphate dikinase (PPDK). Finally, PEP is exported to the mesophyll cytosol to serve as CO_2 acceptor. Simplified, malate generated from PEP can be considered as a bucket for CO_2, and a bucket chain of metabolites shuffles CO_2 from mesophyll cells to bundle sheath cells. In addition, malate also serves as a vehicle for the transport of redox equivalents from mesophyll to bundle sheath cell chloroplasts. The transport processes involved in the C_4-CCM are summarized in Fig. 2.4.

The plastidic electron transfer chain in bundle sheath cell plastids is limited to photosystem I, thus abolishing photosynthetic O_2 production in these cells. The enrichment of CO_2 by malate decarboxylation and minimization of O_2 allows photosynthesis to proceed more efficiently because the oxygenation reaction of Rubisco is minimized due to high CO_2 and low O_2 partial pressures.

CO_2 fixation by Rubisco in bundle sheath cells yields two molecules of phosphoglycerate (3-PGA). The reduction of two 3-PGA to two triose phosphates requires two NADPH. However, the oxidative decarboxylation of one malate yields only one NADPH (and linear electron transport is insignificant in bundle sheath cells), hence one molecule of 3-PGA has to be exported to the MC plastids where it can be reduced to triosephosphate (TP). Two thirds of the generated triosephosphate then need to be re-exported to bundle sheath plastids for regeneration of ribulose-1,5-bisphosphate; the remainder can be exported as sucrose to sink tissues.

From above-said, it becomes obvious that C_4 photosynthesis is dependent on the exchange of malate, oxaloacetate, pyruvate, 3-PGA, and triosephosphate between

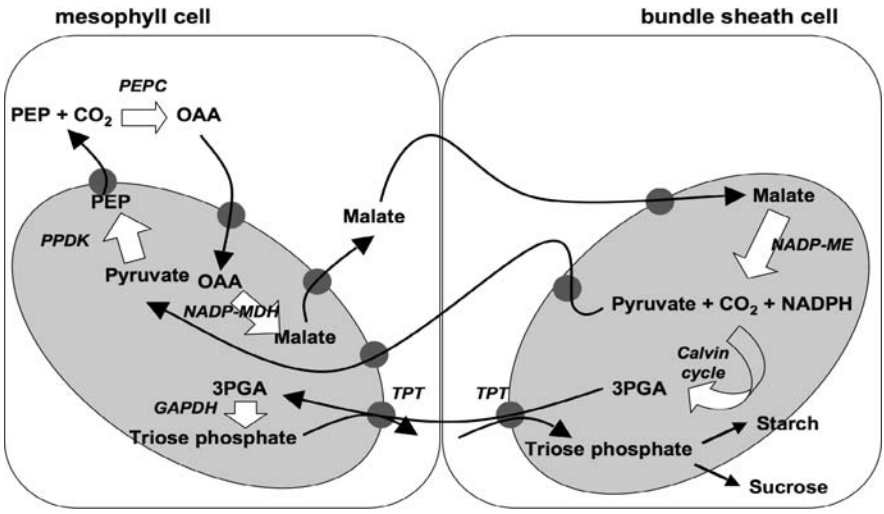

Fig. 2.4 Simplified, schematic representation of transport steps involved in the $NADP^+$-malic enzyme type C_4 carbon-concentrating mechanism

mesophyll and bundle sheath cells and their plastids. It has been hypothesized that symplastic diffusion relying on a concentration gradient accounts for the flux between both cell types. Stitt and Heldt found that gradients for 3-PGA, triosephosphate, and malate were steep enough to drive the required metabolite fluxes between mesophyll and bundle sheath cells [140]. The gradient for pyruvate, however, was minimal and even opposing the direction of transfer. It was proposed that pyruvate could be sequestered to a cellular compartment, most likely the plastids, thereby decreasing the cytosolic concentration and thus generating a concentration gradient between BSC and MC [140]. Weiner and Heldt later determined the subcellular metabolite concentrations and largely corroborated the earlier results [170]. When modeling C_4 photosynthesis, Laisk and Edwards assumed gradients for malate, pyruvate, 3-PGA, and triosephosphate although a gradient for pyruvate was never directly demonstrated [88]. This is likely the reason why the model was not affected by including active pyruvate uptake into plastids. The Laisk–Edwards model succeeds in reproducing data and trends generated earlier by Leegood and von Caemmerer [90]. The maximum carbon (C) fixation rate in their model is 55 μmol C per m^2 leaf area and second, well within the range of experimentally determined values. Maximal carbon fixation capacity may vary between leaf samples as do malate concentrations and transport rates. The enrichment of CO_2 in bundle sheath cells is dependent on overcycling, a higher fixation rate in the MC as compared to the BSC. Overcycling has been calculated and estimated experimentally to be about 10%. A fixation rate of 55 μmol/m^2s and overcycling of 10% thus requires a metabolite flow of ~60 μmol/m^2s: (1) OAA, pyruvate, and 3-PGA into mesophyll plastids; malate, PEP, and triosephosphate out of mesophyll plastids; (2) malate and triosephosphate into bundle sheath plastids; pyruvate and 3-PGA out of bundle sheath plastids. These fluxes are well above those needed to sustain C_3 photosynthesis. As a specific example, the phosphate/triosephosphate translocator (TPT), which is the most abundant protein in C_3 plant plastid envelopes accounting for as much as 10–15% of total envelope protein, catalyzes a maximum flux of 5 μmol substrate/m^2s [48, 49, 54] and TPT limits maximal photosynthetic capacity in C_3 plants [63, 64]. Hence, it is reasonable to hypothesize that transporters involved in C_4 photosynthesis are likely very abundant proteins.

The transporters catalyzing the flux of C_4 photosynthetic intermediates are mostly unknown. Intercellular transport is likely driven by diffusion [140, 170]; thus, there are no apparent requirements for specific transporters at the plasma membrane. In addition, mesophyll and bundle sheath cells in C_4 species are connected by an unusually high number of plasmodesmata.

The intracellular transport of triose phosphate and 3-PGA into and out of plastids is thought to occur via the triose phosphate/phosphate translocator (TPT). This transporter has initially been characterized from spinach [50] and was subsequently found in all plant species that fix carbon, including maize [113]. TPT catalyzes the strict counter-exchange of triose phosphate with 3-PGA or inorganic phosphate.

A PEP/phosphate translocator (PPT) from maize has been demonstrated in isolated maize chloroplasts by Huber and Edwards [72]. A gene encoding a PPT from maize was characterized later [47]. This gene is highly expressed in maize endosperm but transcript abundance was very low in leaves [47], making it unlikely that this particular transporter is involved in the high flux of PEP in the C_4 pathway.

After PEP is exported, OAA is formed by PEPC. OAA levels in maize are too low to be measured, indicating a very rapid conversion of OAA to malate, which can accumulate in considerable amounts. As MDH is localized in plastids, OAA needs to be imported efficiently. Hatch and colleagues have described a high-affinity OAA transporter (OAT) with a K_m for OAA in the range of 0.05–0.07 mM and a corresponding K_i for malate of about 7 mM [59]. The V_{max} was very high in C_4 plastids of maize compared to C_3 plastids from spinach, although K_m and K_i values were similar, indicating that the transporter protein is much more abundant in C_4 plants. We and others have recently demonstrated that the plastidic 2-oxoglutarate/malate translocator DiT1 is able to characterize the specific counter-exchange of OAA with malate [124, 141, 142, 160]. However, the K_i values for malate determined with recombinant DiT1 from maize and spinach are one order of magnitude higher than those determined by Hatch et al. for the OAT, hence DiT1 might not be identical with the OAT that was characterized by Hatch et al. [124, 142].

The export of malate from mesophyll plastids occurs in counter-exchange with oxaloacetate by OAT [59]. However, the malate importer of bundle sheath plastids has not been characterized biochemically or at molecular level to date. For mesophyll cells, a 1:1 stoichiometry for the exchange of malate with OAA fits with the biochemistry of the pathway; however, in bundle sheath cells, for one malate that goes in, one pyruvate goes out. It is thus reasonable to hypothesize the presence of a malate/pyruvate exchanger in bundle sheath chloroplasts of maize. Alternatively, two distinct uniporters could be posited – one that catalyzes the uptake of malate into chloroplasts, and a second one that exports pyruvate.

Pyruvate transport has been characterized in isolated maize mesophyll cell plastids by Huber and Edwards [71]. Later, Flügge et al. analyzed pyruvate uptake into maize leaf plastids and found that the uptake of pyruvate is protein dependent and also dependent on the proton gradient across the inner envelope membrane generated by light [55]. Kanai's group, however, has reported that pyruvate transport, in some C_4 species, is dependent on a sodium gradient [4]. The transport rates of both OAA and pyruvate were calculated and are sufficient to account for the metabolite fluxes during maize photosynthesis [55, 59].

In summary, the transport of 3-PGA and triose phosphates can be accounted for by ZmTPT which is present in large amounts in maize leaf plastids. OAA uptake and pyruvate uptake transporter have been characterized on the biochemical level but their molecular nature is unknown to date. The malate import system of bundle sheath plastids is unknown. A PEP transporter from maize has been reported but its expression pattern argues against a role in C_4 photosynthesis. Overall, most plastidic transporters involved in C_4 photosynthesis are not known at the molecular level.

2.4 Sticking Together – Metabolons and Metabolic Channeling

Compartmentation and distribution of pathways across several cellular organelles is not the only obstacle for constructing networks of plant metabolism. Frequently, several enzymes catalyzing consecutive steps of a multistep pathway are organized

as macromolecular complexes in which metabolic intermediates are passed on from the active site of one enzyme to the next in the cascade, without ever reaching the bulk aqueous phase of the cell. This process is called metabolic or substrate channeling, and the macromolecular complexes formed by soluble enzymes and other polypeptides, such as transporters or scaffolding proteins, are commonly termed "metabolons"; the concept metabolic channeling is not restricted to multienzyme complexes, but it can also occur between different cells or between cellular compartments [77, 172]. While the concept of metabolic channeling is discussed in the literature for half a decade now, the term metabolon is a relatively new one and was originally applied to enzymes of the citric acid cycle that form interactions with the inner mitochondrial matrix [138]. In slightly damaged (permeabilized) mitochondria, these particles show, in comparison to solubilized enzymes, kinetic advantage in converting malate to citrate and in fumarate oxidation [138].

Metabolic channeling is particularly interesting from the perspective of cellular compartmentation. The association of enzymes with macromolecular complexes forms "micro-compartments" within cellular compartments, thus allowing substrates and intermediates to be isolated from the surrounding "macro-compartment" [136]. Hence, distinct pools of metabolites can exist in parallel within the same compartment, thus increasing local substrate concentrations above bulk-phase substrate concentrations and thereby permitting high metabolic rates with low overall bulk concentrations of substrates and intermediates [136]. Also, metabolic intermediates are secluded from competing enzymatic reactions; unstable intermediates are protected; and the release of toxic intermediates to the bulk phase is prevented. A further advantage of metabolic channeling is the increased potential for metabolic regulation by (reversible or temporary) association of enzymes with complexes, which is of particular importance in plant secondary metabolism [2, 76, 77, 172]. It also has been proposed that metabolic channeling and the organization of primitive metabolites and catalysts into ordered metabolic complexes predated the evolution of cells and that cellular life originated from those ordered metabolic complexes [45]. From the perspective of kinetic modeling of metabolic networks, the organization of metabolism into metabolons is problematic, since kinetic constants determined in vitro with purified enzymes cannot be applied to the in vivo situation. However, metabolons, such as the glycolytic subcompartment in heart muscle cells, have been included in mathematical multicompartment models of cardiac metabolism, and it has been demonstrated that the model accurately predicted experimental observations, such as rapid activation of glycolysis and lactate production at the onset of ischemia [182].

2.4.1 Substrate Channeling and Membrane Transport

Metabolic channeling is not exclusive to reactions catalyzed by soluble enzymes, such as steps of arginine biosynthesis [1], cysteine biosynthesis [13], or the degradation of branched-chain amino acids [74]. Also, membrane transport steps can

be involved in the formation of metabolons and association of membrane transporters with soluble enzymes. For example, it was shown that in red blood cells, the Cl^-/HCO_3^- anion exchanger forms an interaction with carbonic anhydrase via binding of carbonic anhydrase II (CAII) to an acidic motif of the transporter's C-terminus [123]. Carbonic anhydrase is thus positioned in close proximity to the cytosolic domains of the transporter, allowing for efficient hydration of CO_2 to HCO_3^- directly at the site of bicarbonate transport. It was shown that attachment of CAII to the transporter accelerates the transport activity by either producing or consuming bicarbonate, depending on the direction of transport [102]. Association of carbonic anhydrase with the bicarbonate transporter and the proton antiporter NHE1 was also demonstrated in renal tissues, and it was shown that the presence of two distinct carbonic anhydrase isoforms on the *cis* and *trans* sites of the renal membrane, respectively, provides the "push" and "pull" for bicarbonate transport [119]. Also, the activity of the monocarboxylate transporter MCT1 is increased when co-expressed with carbonic anhydrase in *Xenopus* oocytes [8]. Another example for the association of metabolic enzymes with a membrane transporter is the association of hexokinase with the voltage-gated anion channel (VDAC) of the mitochondrial outer membrane. Binding of hexokinase to the mitochondrial VDAC is associated with changes in VDAC structure and in interaction with the adenine nucleotide transporter (ANT) in the inner mitochondrial membrane [159]. Association of hexokinase with VDAC prevents apoptosis by preventing the formation of a mitochondrial transition pore consisting of VDAC and ANT working as a uniporter [6, 11]. Association of hexokinase with mitochondria was also recently shown for Arabidopsis [35]; it was previously shown that hexokinase can be associated with the chloroplast outer envelope membrane [171]. Not only hexokinase but also other glycolytic enzymes such as enolase are tightly associated with the outer mitochondrial membrane in yeast [22] and Arabidopsis [57]. In Arabidopsis, 7 of the 10 glycolytic enzymes were found by proteomics in the mitochondrial outer membrane fraction, and it was shown that the entire glycolytic pathway is associated with mitochondria by enzymatic activity assays [57]. Tracer analysis using ^{13}C-glucose demonstrated that isolated, purified mitochondria were able to convert glucose into intermediates of the tricarboxylic acid cycle, providing further evidence for the association of glycolysis with the mitochondrial surface [57]. In yeast, it was shown that enolase is part of a large macromolecular complex that contains glycolytic enzymes, metabolite transporters of the mitochondrial carrier family, and enzymes of the TCA cycle [57].

2.4.2 Metabolic Channeling in Photorespiratory Metabolism in Peroxisomes

A particular good example for metabolic channeling and organization of multiple enzymes as a metabolon represents the arrangement of enzymes of the photorespiratory C_2 oxidation pathway (photorespiration) in leaf peroxisomes [68, 125].