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Plastid transport and metabolism of C_3 and C_4 plants — comparative analysis and possible biotechnological exploitation

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Recent progress in genomics has provided complete or near complete genome sequences of several C_3 (e.g. Arabidopsis, rice, and poplar) and C_4 (e.g. sorghum and maize) plant species. These genome sequences enabled comparative quantitative proteomic and transcriptomic analyses of C_3 and C_4 plants, in particular of their chloroplasts. Such analyses have revealed a comprehensive picture of the distribution of C_4 pathway components between bundle sheath and mesophyll cell chloroplasts and they permitted the prediction of novel pathway components. A comprehensive understanding of the C_4 photosynthetic mechanism is required for the transfer of C_4-like photosynthesis into C_3 crop plants, such as rice.

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Introduction

C_4 photosynthesis is characterized by a biochemical CO_2 pump that increases the concentration of CO_2 at the side of Rubisco, thereby reducing the rate of photorespiration and increasing net CO_2 assimilation [1]. This leads to highly efficient photosynthesis and some of the most productive crops and weeds on earth belong to the C_4 plants, which makes this mode of photosynthesis a desirable trait for introducing it into C_3-type crop plants, such as rice [2••]. C_4 plants can be classified into several biochemical subtypes based on the site and enzymatics of the decarboxylation reaction (see Box 1 for details). In this review, we focus on the metabolite and CO_2 transport processes associated with C_4 photosynthesis in the context of ongoing efforts to introduce this mode of photosynthesis into C_3 plants.

CO_2 assimilation in C_4 plants requires substantially more transport processes than in C_3 plants

C_3 plant chloroplasts contain a complete Calvin cycle. They are thus able to assimilate CO_2 and convert it to the principle product of the Calvin cycle, triose phosphates (TPs). Three molecules of CO_2 have to be assimilated to generate one molecule of TP. Once TP has been generated, it either leaves the chloroplast via the triose phosphate translocator (TPT) in exchange for *ortho*-phosphate or it remains in the chloroplast stroma for the completion of the Calvin cycle or to be converted to transitory starch [3]. Thus, in C_3 plants, at most one transport process across the chloroplast envelope is required per three molecules of CO_2 assimilated.

This is very different in C_4 plants in which, with few exceptions [4], the assimilation of CO_2 is distributed over two cell types, the mesophyll cells (MCs) and bundle sheath cells (BSCs) [1]. The distribution of CO_2 assimilation over two distinct cell types requires a massive flux of metabolites between MCs and BSCs [5,6••]. Each molecule of CO_2 entering a MC is first converted to bicarbonate and then incorporated into phospho*enol*pyruvate (PEP) by PEP carboxylase (PEPC), yielding the C_4 acid oxaloacetate (OAA; see Box 1 for details). The acceptor PEP is generated from pyruvate in MC chloroplasts. That is, pyruvate is taken up into MC chloroplasts by an unknown transporter and converted to PEP by pyruvate:phosphate dikinase (PPDK). PEP then leaves the chloroplasts by a PEP/phosphate translocator (PPT). Depending on the biochemical C_4 subtype (see Box 1), OAA resulting from PEP carboxylation is either converted into the amino acid aspartate in the cytosol of MCs (NAD-ME and PEP-CK subtypes) or to malate in the MC chloroplasts (NADP-ME subtype). For the latter, OAA is imported into the MC chloroplasts, reduced to malate by chloroplast NADP malate dehydrogenase and subsequently exported back to the cytosol, possibly by the dicarboxylate transporter DiT1 [7,8•,9]. Hence, just the prefixation of one molecule of CO_2 in NADP-ME subtype MCs requires already four transport steps across the chloroplast envelope: firstly, import of pyruvate; secondly, export of PEP; thirdly, import of OAA; and fourthly, export of malate.

After the C_4 acid has been generated in the MCs, it is shuffled to the BSCs, likely by diffusion through abundant plasmodesmata, along a steep concentration gradient between MCs and BSCs [10]. Because of space constraints, the following discussion is limited to the NADP-ME subtype only. Malate is taken up into the

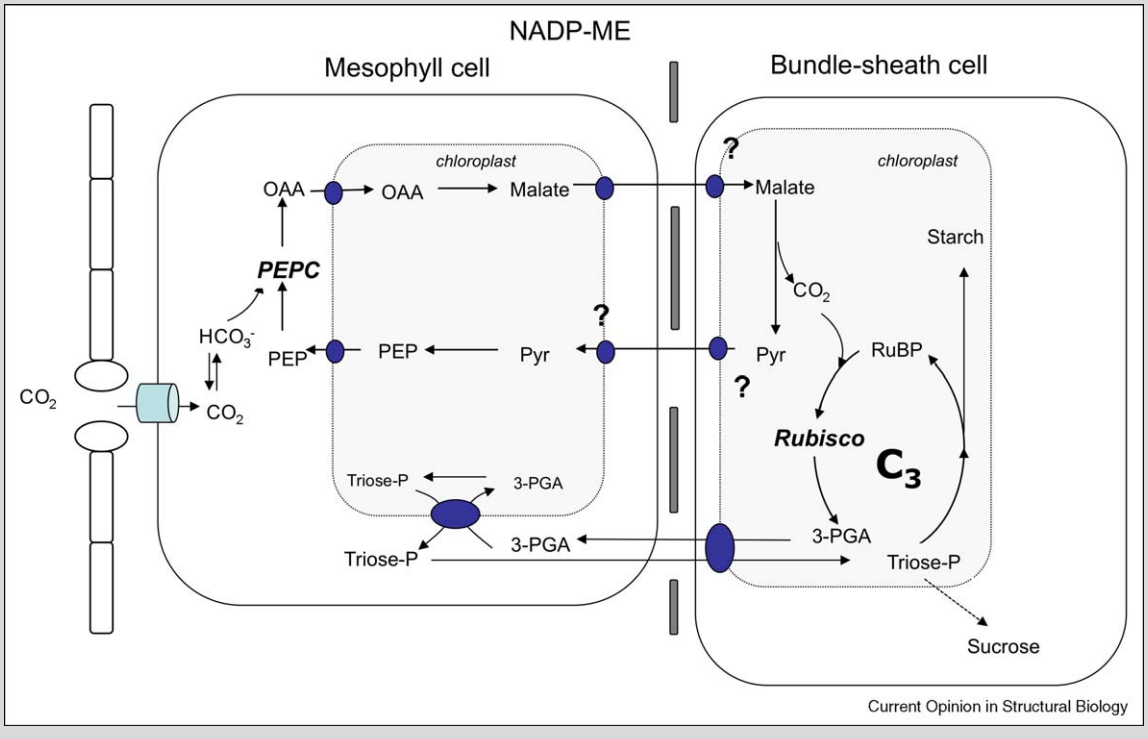
BSC chloroplasts by an unknown transporter and decarboxylated by NADP malic enzyme (NADP-ME), yielding CO_2, NADPH, and pyruvate. CO_2 is assimilated by Rubisco and the pyruvate resulting from oxidative malate decarboxylation is exported to the BSC cytoplasm by an unknown transporter. This adds two more transport steps: firstly, uptake of malate into BSC chloroplasts and secondly, export of pyruvate from these chloroplasts. Pyruvate is returned to the MC for the regeneration of the primary CO_2 acceptor PEP.

The assimilation of one molecule of CO_2 in the Calvin cycle requires two molecules of NADPH since the carboxylation of ribulose 1,5-*bis*phosphate (RuBP) yields two molecules of 3-phosphoglyceric acid (3-PGA) that both have to be reduced to TPs in the Calvin cycle. However, as outlined above, the oxidative decarboxylation of malate in BSCs yields only one molecule of NADPH. Since BSC chloroplasts of NADP-ME subtype C_4 plants have little or no photosystem II activity [11••], they are not able to generate the second required NADPH molecule by linear photosynthetic electron transport. Therefore, one of the two 3-PGA molecules resulting from RuBP carboxylation is exported from BSC chloroplasts, transported to the MCs and taken up into MC chloroplasts (Figure 1). There it is reduced to TP by glyceraldehyde phosphate (GAP) dehydrogenase (GAPDH) and the resulting GAP is exported to the MC cytosol. GAP is transported to the BSCs again and taken up into the BSC chloroplasts for the completion of the Calvin cycle or to be converted to transitory starch. All these transport steps are catalyzed by the TPT in MC and BSC chloroplasts. This adds four more transport steps to the C_4 cycle: firstly, export of 3-PGA from BSC chloroplast; secondly, uptake

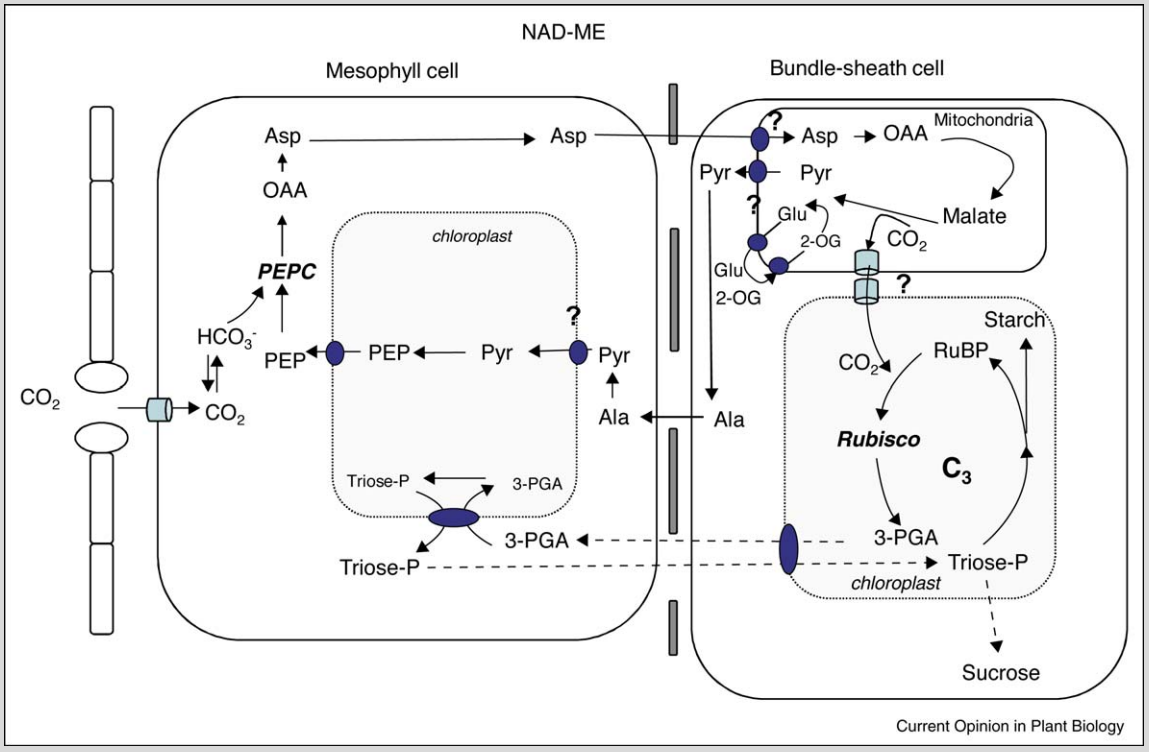
Box 1 Three subtypes of C_4 photosynthesis

Dual-cell type C_4 photosynthetic plants can be classified into three biochemical subtypes, based on the mode and site of decarboxylation of the C_4 (amino) acid. Common to all three subtypes is the initial fixation of CO_2 in the form of bicarbonate by phospho*enol*pyruvate carboxylase (PEPC) in the cytosol of mesophyll cells (MCs). The fate of the resulting C_4 dicarboxylic acid oxaloacetate (OAA) differs, depending on C_4 subtype:

NADP-ME-type C_4 plants: In plants of the NADP-ME subtype, OAA is imported into the MC chloroplasts and reduced to malate. Malate leaves the MC chloroplast and diffuses into the bundle sheath cells (BSCs) along its concentration gradient. In BSCs, malate is taken up into the chloroplasts and oxidatively decarboxylated by the NADP-dependent malic enzyme (NADP-ME). This reaction yields one molecule of each, CO_2, NADPH, and pyruvate. CO_2 is assimilated by Rubisco, yielding two molecules of 3-PGA that can be entered into the Calvin cycle, either in BSCs or in the MCs. The latter requires shuttling of 3-PGA and TP between BSCs and MCs. Pyruvate leaves the BSCs by an unknown transporter, is returned to the MCs and taken up into MC chloroplasts by an unknown transporter. Pyruvate is converted to PEP by pyruvate:phosphate dikinase (PPDK). This reaction consumes ATP and Pi and releases one molecule of each, AMP and pyrophosphate (PPi). Pyrophosphate is cleaved to yield two molecules of Pi by pyrophosphatase. Adenosine monophosphate kinase (AMK) converts one molecule of each AMP and ATP into two molecules of ADP. These can then be converted to ATP by photophosphorylation. PEP leaves the chloroplast by the PEP/phosphate translocator PPT and can enter a new cycle of the carbon dioxide shuttle.



NAD-ME-type C_4 plants: In NAD-ME-type C4 plants, OAA is converted to aspartate by aspartate aminotransferase (AspAT), using glutamate as amino group donor. Aspartate enters the BSCs and there into the mitochondria, where it is converted to OAA by mitochondrial AspAT. OAA is reduced to malate by mitochondrial malate dehydrogenase and malate is subsequently oxidatively decarboxylated by mitochondrial NAD-malic enzyme, yielding NADH, pyruvate, and CO_2. CO_2 enters the Calvin cycle in BSC chloroplasts whereas pyruvate leaves the mitochondria by an unknown transporter. Pyruvate is converted to alanine in BSCs by cytosolic alanine aminotransferase (AlaAT). Alanine enters the MCs, is deaminated to pyruvate by MC AlaAT, yielding pyruvate. Pyruvate is then used to regenerate the acceptor PEP as described above for NADP-ME-type C_4 plants. It is important to note that the Asp/Ala shuttle between MCs and BSCs maintains the ammonia balance between both cell types. Efficient transport of CO_2 between the site of decarboxylation in mitochondria and the site of its assimilation in chloroplasts is essential. It is however not known whether this is protein-mediated or happens by passive diffusion through membranes.



of 3-PGA into MC chloroplasts; thirdly, release of TP from MC chloroplasts; and fourthly, uptake of TP into BSC chloroplasts (Figure 1).

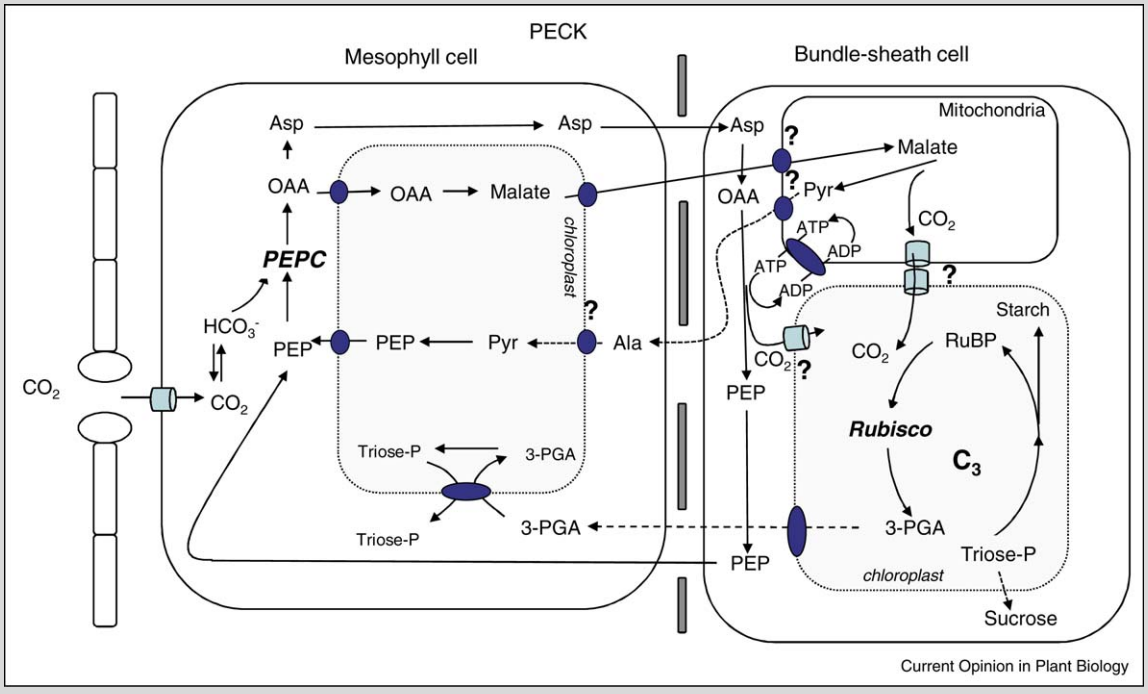
It becomes apparent that the assimilation of a single CO_2 molecule in NADP-ME C_4 plants requires at least 10 transport steps across the chloroplast envelope membranes of MC and BSC. Thus, to produce one molecule of TP for sucrose or transitory starch biosynthesis, at least 30 transport steps are required, in comparison to only one such step in a typical C_3 plant chloroplast. On top of these basic pathway requirements, some 'overcycling' is needed to permit the function of the C_4 biochemical CO_2 pump [12,13]. In addition, C_4 plants typically show higher apparent CO_2 assimilation rates than C_3 plants [14], which further increases the rate of metabolite flux across chloroplast membranes in C_4 in comparison to C_3 plants.

Since metabolite transporters, in contrast to channels and pores, are characterized by relatively low turnover numbers (typically in the range of 10–100 molecules per second and transporter protein), the high flux C_4 photosynthetic pathway likely requires heavy investment into transporter proteins, in particular of those residing in the chloroplast and/or mitochondrial membranes (depending on C_4 subtype). Support for this hypothesis comes from the fact that the TPT of C_3 plants operates close to saturation and becomes flux-limiting under conditions of maximal photosynthesis [15,16].

Implications for engineering a C_4-like photosynthesis in C_3 plants

On the basis of the considerations outlined in the previous section, it is reasonable to assume that engineering a C_4-like photosynthetic pathway into C_3 plants will require increased transport capacity for pathway intermediates across the chloroplast envelope membranes. However, such efforts are hampered by the fact that only two of the required transporters have been unequivocally identified, the TPT and the PPT [6••]. A candidate protein for the MC OAA/malate antiporter has been identified [7,9,17]. However, the kinetic constants of recombinant reconstituted DiT1 proteins from spinach, maize, and *Flaveria*

PEP-CK-type C_4 plants: The PEP-CK-type of C_4 photosynthesis contains elements of the other two subtypes in combination with a different mode of decarboxylation and regeneration of the acceptor. Similar to the NAD-ME-type, a part of the OAA resulting from the PEPC reaction is converted to Asp in MCs and then transported to the BSCs. There, Asp is deaminated to OAA in the cytosol and subsequently decarboxylated by PEP carboxykinase (PEP-CK), yielding PEP and CO_2 at the expense of ATP that is provided by mitochondrial respiration. PEP is then returned to the MCs for a new cycle of CO_2 prefixation. Part of the OAA in MCs, however, is entering the chloroplast where it is converted to malate, similar to the NADP-ME-type C_4 plants. Malate leaves the MC chloroplasts, enters the BSCs and there into the mitochondria for decarboxylation by NAD-ME. The important difference to NAD-ME plants is that malate import into BSC mitochondria leads to a net gain of NADH, which can be used for the production of ATP that is required to drive the cytosolic PEP-CK reaction. Hence the malate shuttle complements the Asp shuttle by shuffling reducing power from MC chloroplasts to BSC mitochondria. The malate shuttle further yields pyruvate, which can be converted to Ala in the BSCs and thereby consumes the amino group introduced to the BSCs by import of Asp. Hence in addition to providing redox power for ATP synthesis, the malate shuttle also contributes to maintaining the ammonia homoeostasis between BSCs and MCs (for simplicity, details of the reaction are not indicated in the diagram). It becomes apparent that the PEP-CK-type of C_4 photosynthesis is slightly more complex than the other types since it requires two complementary metabolite cycles between BSCs and MCs to maintain energy and ammonia homoeostasis.



It is important to note that various combinations of the above-described pathways occur in nature. For example, maize uses a combination of NADP-ME and PEP-CK-type C_4 photosynthesis.

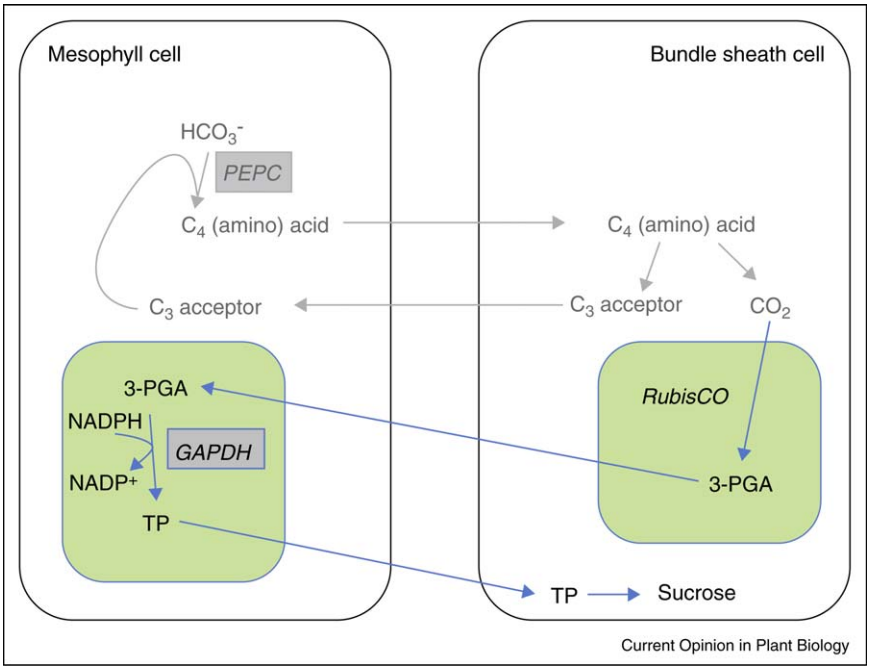
trinervia are not identical with those determined with isolated maize chloroplasts [7,18] since OAA uptake into isolated maize chloroplasts shows only minor inhibition by high concentrations of malate, whereas OAA uptake into isolated C_3 chloroplasts is quite sensitive to malate inhibition [18]. Nevertheless, DiT1 is abundantly expressed in leaves of the C_4 plant maize [9] and sorghum [7] and its expression is specific for MCs [7,8,11••]. It thus remains the best candidate for the MC OAA/malate antiporter.

Pyruvate transport into MC chloroplasts has been studied with a range of C_4 species and two types of transporters could be identified: firstly, a proton:pyruvate cotransporter and secondly, a sodium:pyruvate cotransporter [19]. While the sodium:pyruvate cotransporter remains unknown, a candidate protein for the proton:pyruvate cotransporter (MEP1) has been identified by comparative quantitative proteomics of pea and maize chloroplast envelope membranes [6••], although the function of this protein remains to be proven. The transporters required in BSCs are mostly unknown. Neither the NADP-ME subtype BSC chloroplast malate importer nor the pyruvate exporter has been identified. The same is true for the aspartate importer and the pyruvate exporter of NAD-ME subtype BSC mitochondria.

Prospects for the identification of transporters required for C_4 photosynthesis

The unique features of C_4 photosynthesis (i.e. distribution of labor between MC and BSC, high metabolic flux) might provide the tools required for the identification of those metabolite transporters required for a functional pathway. Since substantially more pathway intermediates must be transported across the organellar envelope membranes of C_4 plants and since transporter

Figure 1



Shuttling of 3-PGA and triose phosphates between bundle sheath and mesophyll cells. Reduction of 3-PGA is shared between bundle sheath and mesophyll cells. Since BSC chloroplast have low PS II activity (at least in the NADP-ME subtype), redox power is scarce in BSC and part of the 3-PGA must be transported to MC chloroplasts for reduction by GAPDH, using NADPH generated by linear electron transport. This possibly also contributes to preventing over-reduction of the MC chloroplasts.

proteins generally show low turnover numbers, it is reasonable to hypothesize that transporters required for the C_4 pathway are more abundant than their orthologous counterparts in C_3 plants [6••]. In addition, since C_4 photosynthesis has evolved multiple times independently in monocotyledonous and dicotyledonous land plants [20], multiple points of reference are available for such comparative analyses. That is, by quantitative comparison of transporter protein abundance (or expression levels of the corresponding genes) in several C_4 biochemical subtypes and C_3 plants, it should be possible to generate a reasonable list of candidate genes for functional studies [6••].

Indeed, a quantitative proteomic comparison of transporter protein abundance in chloroplast envelope membranes isolated from the C_3 plant pea and the C_4 plant maize has identified several membrane transporters of unknown function that are more abundant in C_4 than in C_3 [6••]. In addition, this study also showed that substrate-specific pores of the outer chloroplast envelope membrane were more abundant in C_4 than in C_3 plants, indicating that an increased metabolic flux across the outer envelope membrane of C_4 chloroplasts must be accommodated by increased amounts of the respective porins [6••]. Additional hints on transporter function come from their cell-specific expression patterns. Detailed proteomic analyses of isolated maize MC and BSC chloroplasts have revealed cell-specific expression patterns of metabolite transporters, such as PPT and DiT1, which are both predominantly expressed in MCs [8•,11••]. Others, such as MEP1, are expressed in both MCs and BSCs, which would be consistent with a role of MEP1 in pyruvate transport (although in MCs it would act as an importer whereas it would serve as an exporter in BSCs) [8•,11••].

Comprehensive and quantitative proteomic analyses require fully sequenced genomes and reliable protocols for the isolation of subcellular fractions, including organelles. In the absence of such information and protocols for the vast majority of C_4 plants, comparative transcriptomic analyses could serve as a tool for the identification of novel transporter candidates. Digital gene expression analyses by next-generation sequencing technologies permit the quantitative genome-wide assessment of steady-state transcript levels without prior knowledge of a genome sequence [21•]. Application of such technologies to multiple pairs of closely related C_3 and C_4 species (i.e. *Cleome spinosa* and *C. gynandra*; *Flaveria pringlei* and *F. bidentis* [22]) in combination with state-of-the-art bioinformatics should permit the generation of lists of transporter genes that are consistently higher expressed in C_4 than in C_3 species. Using cell-free expression systems in combination with reconstitution of recombinant transporter protein into liposomes [23] will permit the functional analysis of transporter proteins with reasonable throughput.

CO_2 transport across the plasma membrane and between compartments

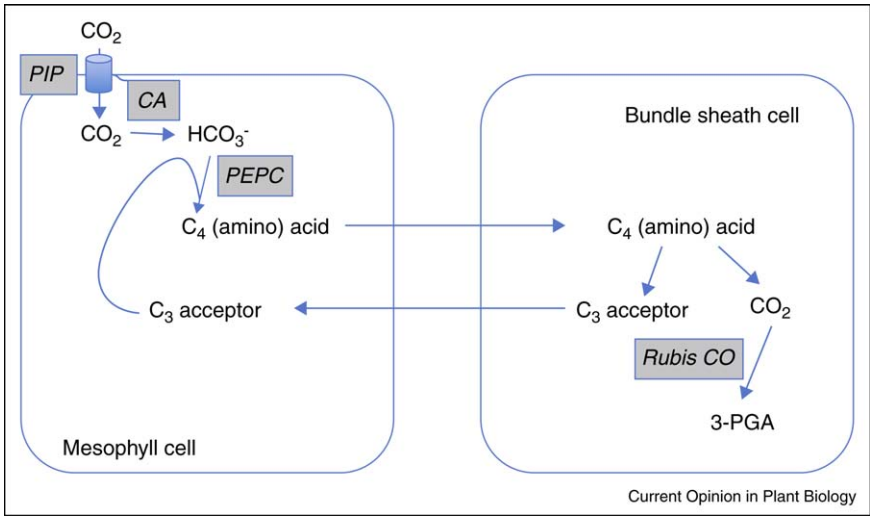
In many cases CO_2 assimilation is limited by the availability of CO_2, which in C_4 species is dependent on the process of diffusion from the atmosphere to the mesophyll cytosol and in C_3 species to the chloroplast stroma. Leaf boundary layer and stomata restrict diffusion to the intercellular airspace. In the liquid phase resistance is in the cell walls, cytosol and the biological membranes. In C_3 species it has been possible to estimate the resistance of the combined liquid path using measurements of carbon isotope discrimination [24]. Evidence for the involvement of porin-mediated pathway for CO_2 diffusion across plasma and inner chloroplast membranes has been given by altering the expression of the aquaporin 1 (Nt AQP1), which belongs to the PIP1 subfamily of aquaporins in tobacco [25••]. In C_4 species high photosynthetic rates require high rates of CO_2 diffusion across the MC plasmalemma, however surface area of mesophyll exposed to intercellular airspace is restricted because of the anatomical constraints of Kranz anatomy [26•] and one might expect to see high porin content in comparison to C_3 in these membranes (see Figure 2 for a hypothetical model). In contrast the need to concentrate CO_2 in BSC would suggest that in this compartment porin content should be low at the plasmalemma in all biochemical subtypes. In NADP-ME species, where CO_2 is released in the chloroplast, these membranes should have low permeability to CO_2 where as in the other subtype (NAD-ME, PCK) where decarboxylation happens outside the chloroplast high permeability is required. The role of porins in CO_2 diffusion remains equivocal and we need to learn more about the role of porins in facilitating CO_2 diffusion and their expression patterns between MCs and BSCs.

Photorespiration in C_3 and C_4 plants

In addition to the metabolite traffic caused by CO_2 assimilation, also intermediates of the photorespiratory pathway have to be transported across the chloroplast envelope membrane [27,28]. This traffic can amount to 30% or more of the apparent rate of CO_2 assimilation [29]. The oxygenation product of RuBP, 2-phosphoglycolate, is dephosphorylated in the chloroplast stroma by phosphoglycolate phosphatase and leaves the chloroplasts to enter the photorespiratory pathway in peroxisomes and mitochondria where two molecules of glycolate are converted into one molecule of glycerate, leading to the release of one molecule of CO_2 [27]. The resulting glycerate is taken up into the chloroplast, phosphorylated to 3-PGA by phosphoglycerate kinase [30], which is then entered into the Calvin cycle. The transporters catalyzing the export of glycolate and the import of glycerate are unknown [27]. The only known transporters involved in photorespiration to date are the plastidic dicarboxylate transporters DiT1 and DiT2, which are required for a functional photorespiratory ammonia cycle [7,17,27].

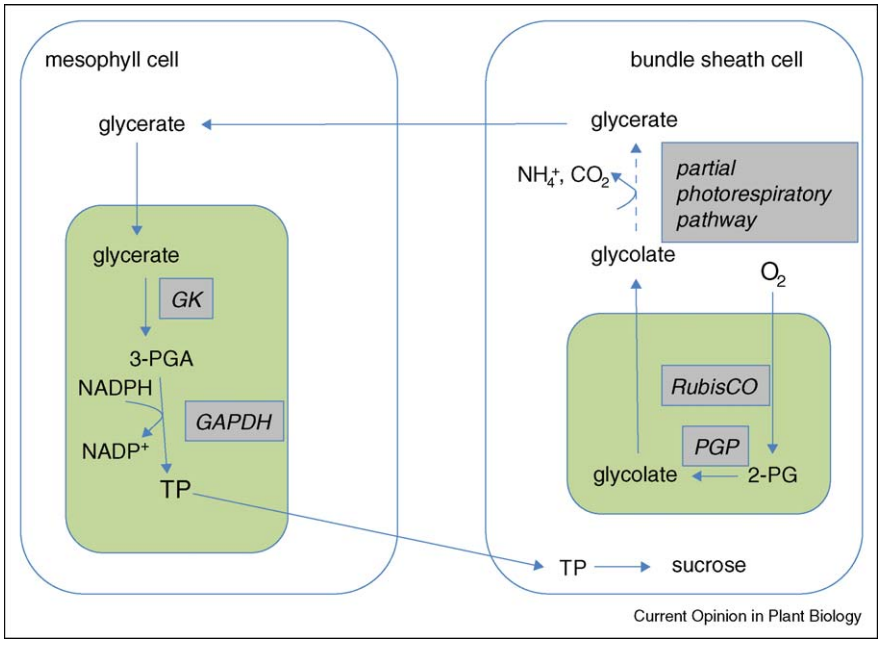
It is frequently assumed that C_4 plants do not perform photorespiration. However, already early studies estimated that the rate of photorespiration in the C_4 plant maize is 2% of that observed in the C_3 plant tobacco [31]. Almost 40 years later, the same author showed that a functional photorespiratory pathway is essential in C_4 plants by demonstrating that maize mutants deficient in glycolate

Figure 2



A hypothetical model of the facilitation of CO_2 diffusion into mesophyll cells. CO_2 diffusion across plasma membrane is facilitated by a CO_2-pore, possibly an aquaporin. We suggest that a membrane-tethered carbonic anhydrase catalyzes rapid conversion to HCO_3^-, which is then incorporated by PEPC into PEP, yielding a C_4 acid. This generates a permanent 'draw' (sink) for bicarbonate, removing it from the pool and thereby promoting influx of fresh CO_2 from the apoplast. The C_4 acid accumulates to high amounts in MC and diffuses into the BSC where it is decarboxylated. Decarboxylation maintains a concentration gradient for C_4 acid, thereby providing the driving force for C_4 acid diffusion.

Figure 3



Photorespiration is distributed between mesophyll and bundle sheath cells in C_4 plants. Recent work using maize mutants showed that an intact photorespiratory cycle is essential for growth in ambient CO_2. Proteomic data and older work showed that most enzymes of the photorespiratory pathway are localized in bundle sheath cells, whereas the last step, glycerate kinase (GK) is located in mesophyll cells. This last step in photorespiration requires the input of redox power (conversion of 3-PGA from GK reaction to triose phosphates). This step is relocated to the compartment with linear electron transport chain generating reducing equivalents from water splitting.

oxidase activity are not viable in ambient CO_2 [32••]. Similar to photosynthetic CO_2 assimilation, also the photorespiratory pathway in the C_4 plant maize is distributed between MCs and BSCs [33]. By quantitative proteomic analysis of isolated maize BSC and MC chloroplasts, it was shown that phosphoglycolate phosphatase is predominantly localized in BSCs [33]. Also the glycolate oxidase and hydroxypyruvate reductase are predominantly localized in BSC [32••,34]. However, in several C_4 species, glycerate kinase, the last enzyme of the photorespiratory pathway, is localized in MC chloroplasts [35]. This makes sense since the reaction product of glycerate kinase, 3-PGA, must be reduced to TP for the regeneration of RuBP, as outlined above for 3-PGA resulting from photosynthetic CO_2 assimilation. Thus, in C_4 plants, photorespiration also contributes to the 3-PGA/TP traffic between BSC and MC (Figure 3).

Conclusions

C_4 photosynthesis requires massive traffic of metabolites across the chloroplast envelope membranes of MCs and BSCs. However, despite many years of research, only few of the genes encoding the transporters required for a functional C_4 pathway have been identified. This lack of knowledge also hampers biotechnological efforts to engineer C_4 photosynthesis into C_3 plants, such as rice [2••]. Considering the massive fluxes of metabolites that are required over organellar membranes and between cell types in C_4 photosynthesis, biotechnological approaches targeting only the soluble enzymes of the pathway appear too simplistic. In addition to metabolite transport, also the transport of CO_2 from the apoplastic space into MCs and beyond is not fully understood. It is likely that CO_2-specific porins, such as CO_2-permeable aquaporins, are required in the plasma membranes of MCs for efficient supply of the C_4 biochemical CO_2 pump with its substrate. We expect that the application of next-generation sequencing technologies to comparative transcriptomics and genomics of related pairs of C_3 and C_4 species, as well as comparative quantitative proteomic analyses will provide us with candidate genes for functional analysis. However, given the complexity of the pathway, even a complete list of all involved enzymes and transporters is unlikely to provide the foundation for a rational engineering of the pathway. For this, it will be crucial to identify the master regulators involved in controlling this complex trait. To this end, a thorough mechanistic understanding of the evolutionary progression from C_3 to C_4 photosynthesis as it occurs in multiple genera of monocotyledonous and dicotyledonous land plants is required.

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- •• of outstanding interest

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