## *Title*
The metabolite transporters of $C_4$ photosynthesis.


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## *Abstract*
$C_4$ photosynthesis is a highly efficient form of photosynthesis that utilises a biochemical pump to concentrate $CO_2$ around rubisco. Although variation in the implementation of this biochemical pump exists between species, each variant of the $C_4$ pathway is critically dependent on metabolite transport between organelles and between cells. Here we review our understanding of metabolite transport in $C_4$ photosynthesis. We discuss how the majority of our knowledge of the transporter families used in $C_4$ photosynthesis has been obtained from studying $C_3$ plants, and how there is a pressing need for *in planta* validation of transporter function in $C_4$ species. We further explore the diversity of transport pathways present disparate $C_4$ lineages and highlight the important gaps in our understanding of metabolite transport in $C_4$ plants. Finally, through integration of functional and transcriptional data from multiple $C_3$ and $C_4$ plants we propose a molecular blueprint for metabolite transport for NAD-ME photosynthesis.


## *Introduction*
Photosynthesis is the process through which plants use solar energy to fix atmospheric $CO_2$ into sugars. Most land plants carry out a form of photosynthesis known as $C_3$

photosynthesis, in which $CO_2$ is fixed into the 3-carbon compound 3-phosphoglycerate (3-PGA) by the enzyme ribulose-1,5-bis-phosphate carboxylase/oxygenase (rubisco). However, the efficiency of $C_3$ photosynthesis is reduced by a competing reaction of rubisco with $O_2$, which produces 2-phosphoglycolate (2-PG), a metabolite that inhibits enzymes of the Calvin Benson Bassham Cycle and starch biosynthesis and must be recycled. While the affinity of rubisco for $O_2$ is much lower than for $CO_2$, atmospheric concentration of $O_2$ is 525 times higher than that of $CO_2$, and thus the ratio of carboxylation:oxygenation in $C_3$ plants is ~3:1[1]. As a consequence, the direct cost of photorespiration, as well as the indirect costs of re-assimilation of released $CO_2$ and $NH_4^+$, are thought to result in losses of up to ~50% of leaf energy[2].

In order to mitigate the cost of photorespiration, several plant lineages have evolved adaptations to increase the concentration of $CO_2$ compared to $O_2$ around rubisco[3–6]. These disparate lineages can be categorised into two different photosynthetic types: CAM (Crassulacean Acid Metabolism) and $C_4$. Both photosynthetic types function by the same principle of initially fixing bicarbonate instead of $CO_2$ using phospho*enol*pyruvate carboxylase (PEPC), an enzyme that is less sensitive to oxygen than rubisco[7]. These species subsequently release and re-assimilate that $CO_2$ using rubisco in a cellular environment where rubisco is protected from oxygen, causing a substantial reduction in photorespiration.

In $C_4$ plants, the separation of initial carbon fixation by PEPC and carbon reduction by rubisco is achieved spatially. $CO_2$ is taken up through open stomata and is fixed by PEPC in mesophyll cells into 4-carbon organic acids. These 4-carbon organic acids are then decarboxylated in a spatially separated location that contains rubisco. Whilst some plants achieve this spatial separation within a single cell[8], in most cases the $C_4$ pathway is split between two specialised cell types, known as the mesophyll and bundle sheath cells (Figure 1). A combination of increased cytosolic distance and/or suberized bundle sheath cell walls

creates diffusive resistance between the site of gas exchange in the mesophyll, and $CO_2$ reduction in the bundle sheath[9]. This reduces the $O_2$ concentration around rubisco while the biochemical pump acts to deliver superatmospheric concentrations of $CO_2$. Accordingly, $C_4$ plants have decreased rates of photorespiration, increased photosynthetic efficiency, and can have increased maximal photosynthetic rates[10]. $C_4$ is also associated with enhanced nitrogen-use efficiency, as less nitrogen needs to be invested in rubisco to achieve the same rate of carbon fixation[11], and enhanced water use efficiency, as the carbon-concentrating effect allows photosynthetic rate to remain high despite low intercellular $CO_2$ concentration caused by stomatal closure[12].

$C_4$ photosynthesis has evolved independently at least 60 times during the evolution of land plants[6]. It is not surprising, given the large number of independent origins, that the $C_4$ trait encompasses significant variation between species. Biochemically, $C_4$ plants are conventionally divided into three subtypes, based on the decarboxylation enzyme with the highest activity in the leaf tissue. These are chloroplastic NADP-malic enzyme (NADP-ME), mitochondrial NAD-malic enzyme (NAD-ME), or cytosolic phospho*enol*pyruvate carboxykinase (PCK)[13]. All 3 subtypes share similar metabolic adaptations in the mesophyll cell. Specifically, in all three subtypes pyruvate entering the mesophyll from the bundle sheath cell is converted to phospho*enol*pyruvate (PEP) by chloroplastic pyruvate, phosphate dikinase (PPDK). Carbonic anhydrase converts $CO_2$ into $HCO_3^-$, and PEPC then generates oxaloacetate (OAA) from $HCO_3^-$ and PEP. OAA is short-lived and spontaneously decomposes back to pyruvate and $CO_2$. Therefore, to stabilise the newly synthesised 4-carbon molecule to enable it to diffuse back to the bundle sheath, OAA is imported into the mesophyll chloroplast where it is converted to either malate (by NADP-malate dehydrogenase (NADP-MDH)), or aspartate (by aspartate aminotransferase (AspAT)), depending on the $C_4$ subtype. If it is stabilised as malate, then it is decarboxylated in the bundle sheath to produce pyruvate and $CO_2$ by chloroplastic NADP-ME (Figure 2A). If OAA

is stabilised as aspartate, then there are two potential routes depending on the primary decarboxylase that is used. In NAD-ME subtype $C_4$ photosynthesis the aspartate is imported into the bundle sheath cell mitochondrion, converted back to OAA by mitochondrial aspartate amino transferase (AspAT), re-stabilised as malate by mitochondrial NAD-MDH and decarboxylated to produce pyruvate and $CO_2$ by mitochondrial NAD-ME (Figure 2B). In PCK subtype $C_4$ photosynthesis, the aspartate is converted to OAA by cytosolic AspAT and then directly decarboxylated to produce PEP and $CO_2$ by cytosolic PCK (Figure 2C).

Although three separate subtypes (NADP-ME, NAD-ME and PCK) have been described, a strict division into these subtypes is now considered to be an over-simplification. Instead many $C_4$ plants possess mixed cycles utilising more than one decarboxylase[14,15]. For example, *Zea mays* (maize) was initially classified as an NADP-ME $C_4$ plant, but has now been shown to carry out an auxiliary PCK pathway[16]. Thus, there is likely a wide range in complexity and diversity within and between $C_4$ species.

***$C_4$ photosynthesis is highly dependent on intracellular metabolite transport***

$C_4$ photosynthesis requires extensive movement of metabolites across the membranes of both chloroplasts and mitochondria. The outer membrane of both organelles contains pores that allow for the non-selective movement of low molecular weight solutes, while the inner membrane contains transporters that control metabolite flux into and out of the organelle[17]. Due to the spatial compartmentalisation of the $C_4$ cycle between the cytosol and organelles, $C_4$ photosynthesis is much more dependent on intracellular metabolite transport than $C_3$ photosynthesis. In $C_3$ photosynthesis, the assimilation of three molecules of $CO_2$ into a molecule of triose phosphate (TP) requires just one intracellular metabolite transport step: the export of TP from the chloroplast via the triose phosphate translocator (TPT) (Figure 3A). In contrast, the assimilation of the same number of molecules of $CO_2$ using the NADP-ME $C_4$ pathway requires at least 31 intracellular metabolite transport steps[18] (Figure 3B). In a conventional NADP-ME $C_4$ pathway these transmembrane transport steps are: the import

of OAA and export of malate from the mesophyll chloroplast, the import of malate and export of pyruvate from the bundle sheath chloroplast, the import of pyruvate and export of PEP from the mesophyll chloroplast, and the exchange of TP and 3-PGA across the envelopes of both mesophyll and bundle sheath chloroplasts. The latter exchange, while not a part of the core $C_4$ cycle, is a necessary feature of $C_4$ plants containing an NADP-ME cycle, and is proposed to be related to the downregulation of photosystem II (PSII) in the bundle sheath of these species[19,20]. This downregulation leads to increased cyclic electron transport around PSI, which produces ATP but not NADPH. The absence of NADPH production assists in kinetically favouring the forward (NADPH-producing) reaction of NADP-ME[21]. However, the reduction stage of the Calvin-Benson-Bassham cycle requires NADPH, and stoichiometrically only half of this can be provided by NADP-ME through decarboxylation of malate[18]. Therefore, half the 3-PGA generated in the bundle sheath chloroplast must be transported to the mesophyll chloroplast to be converted to TP, through the actions of phosphoglycerate kinase (PGK) and glyceraldehyde 3-phosphate dehygrogenase (GAPDH), utilising NADPH produced by linear electron transport in the mesophyll[22]. TP must then be returned to the bundle sheath chloroplast for the completion of the Calvin cycle[18].

Despite the importance of intracellular metabolite transport for $C_4$ photosynthesis, our knowledge of the transporters responsible for these transport steps lags behind our knowledge of the enzymes involved in the $C_4$ cycle. Here we review our current knowledge of these transporters. We discuss how these $C_4$ cycle transporters have been co-opted from ancestral functions in plant metabolism, and how experimental validation is needed to confirm function in $C_4$ plants. We also highlight the major gaps in our understanding of metabolite transport in $C_4$ photosynthesis and propose a complete transport model for NAD-ME $C_4$ photosynthesis.

***Chloroplastic dicarboxylate transport – the DCT family***

Transport of dicarboxylates across the chloroplast envelope occurs primarily via transporters belonging to the divalent anion-sodium symporter (DASS) family. Although this family contains both symporters and antiporters, it is named after the $Na^+$:dicarboxylate symport mechanism exhibited by most of its bacterial and mammalian members[23]. To date, all characterised members of the dicarboxylate transporter (DCT or DiT) subfamily from plants function as chloroplast-localised dicarboxylate antiporters, and were first identified in pursuit of transporters involved in chloroplast nitrogen metabolism[24–26].

Photorespiratory nitrogen assimilation via the GS/GOGAT (glutamine synthetase/glutamine oxoglutarate aminotransferase) pathway in $C_3$ plants requires the exchange of 2-oxoglutarate (2-OG) for glutamate at the chloroplast envelope[27]. The first characterisation of this activity came from the observation of the release of pre-loaded radio-labelled dicarboxylates (malate, succinate, or aspartate) from isolated spinach chloroplasts, in exchange for external dicarboxylates (malate, succinate, aspartate, glutamate, 2-oxoglutarate, fumarate, or OAA)[28]. Determination of the transport kinetics for these different dicarboxylates suggested the existence of two separate exchangers of overlapping specificity: one that preferentially transports aspartate, and one that preferentially transports non-amino acid dicarboxylates (Figure 4A) [29] [27,30,31]. The purification and kinetic characterisation of a 2-OG/Malate transporter (OMT1, sometimes referred to as DiT1), that is unable to transport amino acids such as aspartate or glutamate[24,25,32,33], matched the first of these hypothesized transporters. Subsequently, a general Dicarboxylate Transporter (DCT, sometimes referred to as DiT2) identified through kinetic analysis of spinach chloroplasts pre-loaded with glutamate[34], matched the second[24,25].

Nitrogen assimilation is not the only cellular process in which dicarboxylate exchange across the chloroplast envelope plays a role. Reducing equivalents generated by photosynthetic electron transport must also be exported to be used in the cell. One mechanism for the

transport of reducing equivalents across the chloroplast envelope is the 'malate valve', in which an OAA/malate exchanger acts in combination with malate dehydrogenases acting in opposite directions on either side of the membrane[35] (Figure 4B). A significantly increased $K_m$ for OAA uptake into chloroplasts isolated from *omt1* mutant *A. thaliana* suggests that OMT1 is the high-affinity OAA transporter that forms part of the malate valve in *A. thaliana* chloroplasts[36]. Interestingly, the dual function of OMT1 in nitrogen assimilation as well as in the malate valve may help integrate these separate pathways. Specifically, during primary nitrogen assimilation, OMT1 may help to coordinate the supply of reducing power to cytosolic nitrate reductase (NR)[25].

In $C_4$ plants, the DCT subfamily proteins have been co-opted from their ancestral roles above to facilitate $C_4$ cycle dicarboxylate exchange across the chloroplast envelope[26]. As is typical for components of the $C_4$ cycle, *DCT* subfamily genes show higher expression in $C_4$ than $C_3$ plants, and this expression is asymmetrically distributed between mesophyll and bundle sheath cells. In maize there are three *DCT* subfamily genes – *OMT1*, *DCT1*, and *DCT2*. *ZmOMT1* is expressed strongly in the mesophyll only, *ZmDCT1* is expressed to a lower level, and preferentially in the mesophyll, and *ZmDCT2* is expressed strongly in the bundle sheath only[26,37] (Figure 4C). This pattern is recapitulated in studies on chloroplast envelope proteomes[19,38,39 40]. It is therefore proposed that ZmOMT1 localised in the mesophyll chloroplast envelope facilitates the exchange of cytosolic OAA with chloroplastic malate as part of the $C_4$ cycle[26] (Figure 4D). In a similar manner, ZmDCT1 localised in the mesophyll chloroplast envelope may facilitate the exchange of OAA with chloroplastic aspartate (generated by AspAT), as part of the auxiliary PCK cycle in maize (Figure 4D). These hypotheses have not formally been tested, and thus the generation and characterisation of *omt1* and *dct1* mutants in $C_4$ plants are necessary to confirm this role.

In contrast to the proposed role of OMT1 in the mesophyll cell chloroplast, ZmDCT2 is proposed to be involved in the import of malate into the bundle sheath chloroplast in the NADP-ME $C_4$ cycle (Figure 4E). This is supported by the analysis of *dct2* mutant maize[41], in which isolated bundle sheath chloroplasts show lower rates of malate uptake compared to those from wild-type plants[41]. Additionally, radio-labelled $CO_2$ and malate feeding experiments (using whole plants and isolated bundle sheath cells respectively) indicate an increased production of aspartate in place of malate: consistent with a diversion in flux from the disrupted NADP-ME $C_4$ cycle to the auxiliary PCK cycle[41]. It should be noted that DCT2, being an obligate dicarboxylate exchanger, cannot by itself facilitate the net import of malate into the bundle sheath chloroplast that is required to run the $C_4$ cycle. Therefore, the model that has been proposed to explain the role of DCT2 in malate import involves the existence of a separate, as-yet-unidentified aspartate uniporter (or cation symporter), which imports aspartate into the bundle sheath chloroplast so that DCT2 can carry out aspartate export in exchange for malate import[41] (Figure 4E). This proposal is consistent with previous observations that malate-dependent pyruvate formation from maize bundle sheath cells or chloroplasts is stimulated by aspartate[42,43].

Although DCT2 is likely involved in malate uptake in maize, different $C_4$ species have subfunctionalised different *DCT* subfamily members. For example, other NADP-ME subtype $C_4$ species *Sorghum bicolor* (sorghum), *Setaria italica,* and *Setaria viridis* show strong bundle sheath-specific expression of *DCT4*, a duplicate of *DCT1* that is not found in maize[44–46] (Figure 4C). In contrast to NADP-ME, the NAD-ME species *Panicum virgatum* (Figure 4C) and *Cleome gynandra*, and the PCK species *Urochloa fusca*, have low expression of *DCT* genes[46–48]. The absence of this transporter from species that do not take up malate into bundle sheath chloroplasts further supports the proposed role of *DCT*2/4 in malate uptake into the bundle sheath chloroplasts of NADP-ME $C_4$ species.

### *Chloroplastic monocarboxylate/phosphate transport – the TPT family*

The primary transport flux of monocarboxylates across the chloroplast inner envelope occurs via the Triose Phosphate Translocator (TPT) family, members of the larger Drug/Metabolite Transporter (DMT) superfamily. TPT family proteins are specific to eukaryotes[49], and catalyse the exchange of phosphorylated monocarboxylates with inorganic phosphate ($P_i$).

#### TPT

The first observation of TPT activity was the exchange of $P_i$ with phosphorylated 3-carbon compounds across the envelopes of isolated spinach chloroplasts[28]. The rate of exchange was high with triose phosphates phosphorylated on the third carbon, lower for those phosphorylated on the second carbon, and lower still for sugar phosphates[28,50]. Given these transport properties, an obvious function for TPT is the export of the primary products of photosynthesis from the chloroplast to the cytosol. Accordingly, in $C_3$ plants TPT plays a direct role in the coordination between sucrose and starch biosynthesis. For example, reduced sucrose biosynthesis, and therefore reduced triose phosphate exchange with $P_i$ through TPT, leads to reduced $P_i$ and increased triose phosphate levels in the stroma, which leads to increased starch biosynthesis via the allosteric activation of chloroplastic ADP-glucose pyrophosphorylase (Figure 5A)[51].

Additional roles for TPT in $C_3$ plants have been proposed. Similarly to the chloroplast malate valve, TPT can act in concert with chloroplastic and cytosolic enzymes to indirectly export reducing equivalents to the cytosol, although in this case TPT also results in the indirect export of ATP[22]. Here, in the chloroplast 3-PGA is converted by the ATP-consuming PGK and NADPH-consuming GAPDH into GAP, whilst in the cytosol GAP is converted by the NADH-producing GAPDH and ATP-producing PGK back to 3-PGA. In this way, both ATP and reducing power are indirectly “shuttled” from the chloroplast to the cytosol (Figure 5B). In $C_4$ plants, this shuttle is distributed across the two cell types to transfer reducing equivalents produced by linear electron transport in mesophyll chloroplasts to bundle sheath

chloroplasts (Figure 5C). Consistent with this role, NADP-ME subtype $C_4$ plants show higher leaf *TPT* RNA and TPT protein levels than $C_3$ plants[40,52], and high *TPT* RNA and TPT protein levels in both mesophyll and bundle sheath[19,37–39,44,45] (Figure 5D). It should be noted that *TPT* is also expressed to high levels in both mesophyll and bundle sheath of NAD-ME subtype $C_4$ plants such as *P. virgatum* (Figure 5D) and *C. gynandra*[48], despite the fact that NAD-ME plants do not show depleted bundle sheath chloroplast PSII activity[53]. It is therefore likely that redox/ATP balancing is required between mesophyll and bundle sheath chloroplasts in all $C_4$ plants, not just in NADP-ME subtype $C_4$ plants where the down-regulation of PSII in the bundle sheath makes this need more apparent.

**GPT and XPT**

It was initially assumed that the exchange of phosphate with triose phosphates and sugar phosphates was carried out by the same broad-specificity TPT carrier. However, plastids from different tissues were found to exchange different phosphorylated compounds for $P_i$ with differing efficiencies[54]. For example, plastids isolated from pea roots and maize endosperm can efficiently exchange $P_i$ with glucose-6P, in order to compensate for their lack of the gluconeogenesis enzyme fructose 1,6-bisphosphatase[55,56]. This exchange reaction is facilitated by a separate TPT-family carrier, the glucose-6P/$P_i$ translocator (GPT)[57] (Figure 6A). In contrast to *TPT*, in $C_3$ plants *GPT* tends to be highly expressed in non-green tissues, where it is proposed to supply plastids with glucose-6P for starch biosynthesis and/or the oxidative pentose phosphate pathway (OPPP)[57]. More recently, another TPT-family member was identified based on sequence similarity to GPT: the xylulose-5P/$P_i$ translocator (XPT). XPT, which is expressed throughout the plant, is an efficient transporter of xylulose-5P and is proposed to connect cytosolic and plastidic reactions within the OPPP[58] (Figure 6A). The potential functions of GPT and XPT in $C_4$ plants have not yet been studied. However, *GPT* genes show high, cell-type specific expression in $C_4$ plants. For example, Maize (NADP-ME), Sorghum (NADP-ME), and *C. gynandra* (NAD-

ME) show bundle sheath-preferential expression[37,44,48], whilst *S. italica* (NADP-ME) shows mesophyll-preferential expression[45] (Figure 6B). This transcriptome signature is indicative of a potential role in $C_4$ photosynthesis and further work in $C_4$ plants needs to be done to elucidate this.

**PPT**

A final member of the TPT family is the PEP/$P_i$ translocator PPT, which is an efficient transporter of triose phosphates that have been phosphorylated on the second carbon, such as PEP and 2-PGA, although a low affinity for 2-PGA means that *in vivo* PEP is likely the main substrate[59]. In *A. thaliana* there are two *PPT* genes: *PPT1* and *PPT2. PPT2* is ubiquitously expressed throughout the leaf, and is hypothesized to carry out the housekeeping role of PEP import into the chloroplast, for entry into the shikimate pathway for the production of aromatic amino acids[60,61] (Figure 7A). *PPT1*, on the other hand, is specifically expressed in the leaf vasculature (and not the mesophyll cells) and the roots[59,60]. PPT1 is also sometimes referred to as CAB (chlorophyll a/b binding protein) UnderExpressed 1 (CUE1), due to its initial identification in an *A. thaliana* mutant screen for regulators of light-responsive genes[62,63]. These plants show a reticulate leaf phenotype caused by impaired mesophyll chloroplast development[62], which in turn is related to a deficit in the PEP-derived aromatic amino acid phenylalanine and downstream phenolic compounds[61,63]. Therefore, it is proposed that PEP transport by PPT1 is involved in the synthesis of an unknown plant secondary metabolite in the leaf vasculature that is not required in the vasculature itself, but diffuses into the mesophyll where it is required for mesophyll cell development[60,61] (Figure 7B). This is consistent with work in tobacco showing that several phenolic compounds are involved in the regulation of cell division and expansion in leaf mesophyll cells[64], and that disruption in phenolic metabolism can lead to a reticulate leaf phenotype very similar to *A. thaliana cue1*[64,65].

In $C_4$ plants of all subtypes, the inverse of the $C_3$ chloroplast envelope PEP transport reaction is required. Rather than PEP import, PEP must be exported from the mesophyll chloroplast to provide the substrate for PEPC (Figure 7D). Usually, an ortholog of *PPT1* has been co-opted for function in the $C_4$ cycle[66], and correspondingly shows higher RNA and protein levels in $C_4$ plants than in $C_3$ relatives[40,47,52,67], as well as preferential expression in the mesophyll[19,37–39,44,45,48] (Figure 7C). Although transport activity and cell-type expression data are highly indicative, PPT function in the $C_4$ cycle has never been directly tested through analysis of *PPT* knock-down or knock-out $C_4$ plants. As well as confirming a role for PPT in the $C_4$ cycle, it would be interesting to investigate if the movement of *PPT1* expression from bundle sheath to mesophyll during the evolution of $C_4$ photosynthesis is related to the morphological adaptations present in $C_4$ plants, given the apparent developmental role played by PPT1 in $C_3$ plants.

***Chloroplastic pyruvate transport – the BASS family***

Although all $C_4$ plants rely on chloroplastic pyruvate uptake, only one family of transporter proteins has been linked to this activity so far. This is the Bile Acid/Sodium Symporter (BASS) family, of the Bile/Arsenite/Riboflavin Transport (BART) superfamily. These families are so-named due to their function in animals of transporting bile salts[68]. However, BASS proteins are also found in prokaryotes and other eukaryotes, including plants. In plants, there are five BASS subfamilies, BASS1-5[69].

***BASS2***

Carrier-mediated pyruvate uptake into chloroplasts was first demonstrated using chloroplasts isolated from the mesophyll cells of the $C_4$ plant *Digitaria sanguinalis*[70]. It was later shown that this transport reaction in maize is light-dependent[71], and in *Panicum miliaceum* that pyruvate uptake is directly dependent on the proton gradient that is generated across the chloroplast envelope under illumination[72,73]. Subsequent sampling across a broader species range revealed that light-dependent chloroplast pyruvate uptake

can be divided into two mechanisms: $H^+$- and $Na^+$-dependent[74]. $H^+$-dependent pyruvate uptake is limited to two sister lineages within the grasses that are both derived from the same origin of $C_4$ photosynthesis[75]: the Arundinelleae and Andropogoneae, the latter of which contains several important $C_4$ crop species such as maize, sugarcane, and sorghum[74,76]. $Na^+$-dependent pyruvate uptake on the other hand was found in all other monocot as well as all dicot clades tested so far[74,77,78].

$Na^+$-dependent pyruvate import is now known to be facilitated by BASS2, in conjunction with $Na^+/H^+$ antiporter 1 (NHD1)[79] (Figure 8). Both *BASS2* and *NHD1* are expressed to higher levels in the leaves of $Na^+$-type $C_4$ species than in close $C_3$ relatives[47,67], and BASS2 protein was similarly found in the leaves of multiple $Na^+$-type $C_4$ species but not $H^+$-type $C_4$ species or $C_3$ species[79]. As expected, *BASS2* and *NHD1* are also preferentially expressed in the mesophyll cells of $Na^+$-type $C_4$ species *S. italica* and *P. virgatum*[45,48] (Figure 8A). It is proposed that NHD1 utilises the light-dependent proton gradient across the chloroplast inner envelope to couple the import of $H^+$ down the proton gradient to the export of $Na^+$ into the intermembrane space. Pyruvate is then imported by pyruvate/$Na^+$ symport through BASS2[79] (Figure 8B). While analysis of $C_4$ *bass2* mutant plants is yet to be performed, the function of BASS2 in $C_3$ plants has been investigated using *A. thaliana bass2* mutant plants. Analysis of these plants led to the proposal that BASS2 acts to import pyruvate into the chloroplast for entry into the MEP pathway for terpenoid biosynthesis (Figure 8C).

***Other BASS genes (BASS1, BASS4, BASS6)***

Other BASS family members with different substrate specificities have also been characterised in plants. In a $C_3$ context, *A. thaliana* BASS1 was demonstrated to act as a chloroplast pantoate transporter involved in pantothenate biosynthesis[80], whilst *A. thaliana* BASS6 was shown to facilitate the export of photorespiratory glycolate from the chloroplast[81], alongside the previously characterised plastidic glycolate/glycerate transporter (PLGG1)[82]. In a $C_4$ context, BASS4 was identified alongside BASS2 as a potential $C_4$ cycle

transporter due to high expression in some $C_4$ dicots compared to $C_3$ relatives (from the *Flaveria* genus)[79], although it is not highly expressed in the $C_4$ dicot *Cleome gynandra*[79] or any $C_4$ monocots tested so far[37,44,45,48]. Thus, whilst BASS4 may be involved in $C_4$ photosynthesis in some lineages, it does not appear to be commonly recruited to the role.

***Mitochondrial pyruvate transport – the MPC family***

Transport of pyruvate across the mitochondrial membrane is facilitated by members of the Mitochondrial Pyruvate Carrier (MPC) family, which are found across the eukaryotes, and catalyse a pyruvate/$H^+$ symport reaction[83,84]. The import of pyruvate from cytosolic glycolysis into the mitochondria for entry into the tricarboxylic acid (TCA) cycle is a common transport reaction found in most eukaryotes. Mitochondrial pyruvate import is also required to support the biosynthesis of branched-chain amino acids leucine and valine (Figure 9A). The first biochemical evidence for a specific pyruvate carrier was obtained in the 1970s[83,85], but it was not until 2012 that two groups independently uncovered the molecular identity of the MPC, primarily through experiments in yeast[84,86]. These experiments showed that in yeast a complex of MPC1 with either MPC2 or MPC3 facilitates mitochondrial pyruvate import to support both the TCA cycle and valine/leucine biosynthesis[84,86]. MPC proteins have subsequently been characterised in the $C_3$ plant *A. thaliana*. The MPC family comprises four members in *A. thaliana*: MPC1, MPC2, MPC3, and an MPC2-like protein called NRGA1 (Negative Regulator of Guard Cell ABA signalling 1). Mitochondria isolated from *mpc1* mutant *A. thaliana* show significantly reduced pyruvate-dependent $O_2$ consumption and pyruvate uptake rates, compared to wild-type[87]. The apparent discrepancy in *mpc1* mutant *A. thaliana* between relatively minor phenotypes (altered drought and cadmium tolerance), and a significant disruption to the crucial supply of pyruvate to the TCA cycle, is explained by the presence of two alternative pathways to indirectly supply mitochondrial pyruvate: namely the import of malate (e.g. via DIC, see below) and oxidation to pyruvate by NAD-

ME, or the import of alanine via an unknown transporter and conversion to pyruvate by AlaAT[87] (Figure 9A).

NAD-ME subtype $C_4$ photosynthesis requires pyruvate transport across the bundle sheath mitochondrial membrane, although here the export of NAD-ME-derived pyruvate is required, in contrast to the characterised pyruvate import activity of MPC. The NAD-ME subtype dicot *C. gynandra* shows significantly upregulated *MPC1* and *MPC3* compared to close $C_3$ relative *C. spinosa*, although not to the extent of other $C_4$ cycle genes[47]. *MPC* genes in *C. gynandra* are also not differentially expressed between mesophyll and bundle sheath[88], as would be expected given the hypothesised bundle sheath-specific $C_4$ function. The NAD-ME subtype monocot *P. virgatum* does show preferential expression in the bundle sheath for *MPC1* and *MPC2*[48]. This is in contrast to NADP-ME subtype monocots, which show only very low bundle sheath expression of *MPC* genes, as would be expected given the lack of mitochondrial malate decarboxylation in this subtype[37,44,45] (Figure 9B). Despite this somewhat promising expression data, there is no experimental evidence to support the hypothesis that MPC can facilitate mitochondrial pyruvate export. In fact, mitochondria isolated from *mpc1* knock-out *A. thaliana* are still capable of exporting pyruvate[87], indicating the presence of another, as-yet-unidentified pyruvate exporter in plants that would also be a candidate for co-option into the $C_4$ cycle. Thus, the identity of the uncharacterised pyruvate exporter and the role of MPC in NAD-ME $C_4$ photosynthesis remains to be elucidated.

***Mitochondrial dicarboxylate transport – the MC family***

Dicarboxylate transport across the mitochondrial membrane is facilitated by several transporters from the Mitochondrial Carrier (MC) family. This is a large family of transporters, found across eukaryotes, with a common structure but highly varied substrate specificity[89]. In relation to $C_4$ photosynthesis, three MC subfamilies have so far been implicated in a role in $C_4$ pathways: the Dicarboxylate Carrier (DIC), Uncoupling Protein (UCP), and Dicarboxylate and Tricarboxylate Carrier (DTC) subfamilies.

**DIC**

DIC proteins carry out the exchange of dicarboxylates (including malate, OAA, and succinate), phosphate, sulphate and thiosulphate[90]. In plants, this transport activity was first observed in isolated bean mitochondria[91]. *A. thaliana* DIC1-3 were identified based on homology to the previously identified human DIC, and transport assays using AtDIC1-3 reconstituted in liposomes demonstrated that these proteins account for this transport activity[90]. Several potential roles for DIC transporters in $C_3$ plants have been proposed (Figure 10A). First, DICs may facilitate the anaplerotic provision of dicarboxylates for the TCA cycle[90], which is consistent with the fact that malate and succinate oxidation in plant mitochondria is inhibited by known inhibitors of DIC[91,92]. Second, DICs may exchange malate and OAA, and act in concert with cytosolic and mitochondrial MDHs to transfer reducing equivalents between cytosol and mitochondria, in an analogous way to OMT1 in the chloroplast[90]. Third, high expression of *DIC1* and *DIC2* in cotyledons may indicate a role in the exchange of OAA and malate with succinate across the mitochondrial membrane that is required during the mobilisation of storage lipids via the glyoxylate cycle and gluconeogenesis[90].

It was initially assumed that the NAD-ME subtype $C_4$ cycle required the mitochondrial import of only aspartate (the 4C transfer acid from the mesophyll) and 2-OG (required for the trans-amination reaction catalysed by AspAT to convert Aspartate into OAA). However, mitochondria isolated from the NAD-ME $C_4$ plant *Atriplex spongiosa* were found to require not only aspartate and 2-OG, but also malate and $P_i$, to sustain maximal rates of pyruvate and $O_2$ formation[93,94]. This process is also inhibited by a known inhibitor of DIC[91,93]. Thus, the exchange of malate and $P_i$ by DIC seems to be required in NAD-ME subtype bundle sheath mitochondria. Transcriptomics data has subsequently supported a role for DIC in the NAD-ME cycle. Among $C_4$ monocots, two paralogs of *A. thaliana DIC3* are highly expressed, in a strongly bundle sheath-preferential manner in the NAD-ME subtype species *P.*

*virgatum*[48], but not in NADP-ME subtype species[37,44,45] (Figure 10B). In dicots, *DIC1* shows significantly bundle sheath-preferential expression in the NAD-ME subtype $C_4$ species *C. gynandra*[88], and this gene is significantly upregulated compared to its close $C_3$ relative *C. spinoza*[47]. In contrast, no *DIC* genes show significant upregulation when comparing NADP-ME subtype $C_4$ species to close $C_3$ relatives within the *Flaveria* genus[52]. The expression patterns above for *DIC* correlate with the expression of genes encoding the mitochondrial Phosphate Carrier (PIC): a $P_i/H^+$ symporter[95]. This suggests a mechanism by which malate import (and $P_i$ export) through DIC is coupled to the proton gradient across the mitochondrial membrane via $H^+$ and $P_I$ symport through PIC (Figure 10C).

**DTC**

DTC proteins catalyse the strict counter-exchange of dicarboxylates (including 2-OG, malate, OAA, succinate) and tricarboxylates (including citrate, isocitrate)[96,97]. DTC proteins are found only in plants and protozoa, although they are closely related to 2-OG/malate carriers found in animals, and were first identified in *P. miliaceum* based on homology to a previously characterised mammalian 2-OG/malate carrier[97]. A role in NAD-ME subtype $C_4$ photosynthesis was immediately hypothesised, based on light-dependent, leaf bundle sheath-specific expression, and developmental regulation similar to other $C_4$ cycle genes[98]. This has subsequently been corroborated with transcriptomics data, with *DTC* genes showing high, bundle sheath-specific expression in NAD-ME[48], but not in NADP-ME subtype $C_4$ monocot species[37,44,45] (Figure 11A). The NAD-ME subtype $C_4$ dicot species *C. gynandra* also shows significantly bundle sheath-preferential expression of *DTC*[88], although the gene is not upregulated in the NAD-ME subtype $C_4$ dicot species *C. gynandra* compared to its close $C_3$ relative *C. spinoza*[47].

The role for DTC transporters in $C_3$ plants has not been well characterised, although multiple hypotheses have been proposed (Figure 11B). First, the capacity for malate/OAA exchange means a redox valve function is possible[96]. Second, the capacity for citrate exchange with

OAA and/or malate could play a role in fatty acid elongation and isoprenoid synthesis[96]. The only hypothesis for which there is currently any experimental evidence is a potential role in nitrogen assimilation. Nitrogen assimilation via the GS/GOGAT cycle in the chloroplast requires the supply of 2-OG carbon skeletons. DTC, through its capacity to exchange various TCA cycle intermediates, may be involved in the direct export of 2-OG produced by mitochondrial isocitrate dehydrogenase to the cytosol, or the export of citrate that can then be converted to 2-OG by cytosolic aconitase and isocitrate dehydrogenase[99]. This is supported by the observation that *DTC* transcript levels in nitrogen-starved *Nicotiana tabacum* plants increased upon the addition of nitrate[96], in a similar pattern to that previously observed for isocitrate dehydrogenase transcripts[100,101].

**UCP**

UCPs are so-named because they were first identified based on their ability to transport $H^+$ in order to dissipate the proton gradient across the mitochondrial membrane and thus uncouple mitochondrial electron transport from ATP synthesis[102]. Whilst six family members were originally identified in *A. thaliana* (*UCP1-6*)[103], UCP4-6 were subsequently found to transport dicarboxylates, and to cluster with DICs from animals and yeast, and were therefore re-classified as DIC1-3 (discussed earlier)[90]. This leaves three UCP members in plants, UCP1-3, which cluster with UCPs from animals and yeast[90].

In plants, UCPs, sometimes called Plant Uncoupling Mitochondrial Proteins (PUMPs), were first identified in mitochondria isolated from potato[104]. This potato UCP, when reconstituted into liposomes, was found to transport $H^+$ [104]. Inhibiting its activity *in planta* led to a significant increase in mitochondrial $H_2O_2$ generation, suggesting a role in uncoupling mitochondrial electron transport in order to protect the mitochondria against oxidative stress[105]. Interestingly, in the context of both $C_3$ and $C_4$ photosynthesis it was recently shown that AtUCP1 and 2, when reconstituted into liposomes, are able to exchange dicarboxylates (such as malate, OAA, 2-OG, succinate), amino acids (such as aspartate and glutamate),

as well as other small compounds (such as sulfate, thiosulfate, and $P_i$)[106]. GC-MS-based metabolic profiling showed that *ucp* mutant *A. thaliana* plants had reduced levels of the organic acids malate, fumarate, and citrate, compared to wild-type[106]. In light of this new biochemical and metabolic evidence, it is suggested that the photorespiratory role of AtUCP1/2 may be as part a redox valve to supply reducing equivalents from photorespiratory, mitochondrial GDC to photorespiratory, peroxisomal hydroxypyruvate reductase. It has already been suggested that reducing equivalents are transferred between mitochondria and peroxisome via a malate/aspartate shuttle, in which glutamate and malate are transferred from mitochondria to peroxisome, with aspartate and 2-OG moving in the opposite direction, and linked by MDH and AspAT in both cellular compartments[107]. Malate/2-OG exchange is likely facilitated by DTC, whilst aspartate/glutamate exchange could be carried out by UCP (Figure 12A). Cleary further work is needed to fully elucidate the roles played by UCPs in $C_3$ plants, particularly with regards to photorespiration.

The discovery that UCPs can transport amino acids and dicarboxylates makes them clear candidates for co-option into the NAD-ME subtype $C_4$ cycle. Indeed, in dicots, *UCP1* shows bundle sheath-preferential expression in the NAD-ME subtype $C_4$ species *C. gynandra*[88], and this gene is significantly upregulated compared to its close $C_3$ relative *C. spinoza*[47]. In contrast, there is no consistent upregulation of *UCP* genes between $C_3$ and NADP-ME subtype $C_4$ *Flaveria* species[52]. In monocots, *UCP* genes show moderately high, bundle sheath-specific expression in the NAD-ME subtype $C_4$ species *P. virgatum*[48], and although *UCP* gene expression is also bundle sheath-preferential in NADP-ME subtype $C_4$ monocots, the expression level is much lower[37,44,45] (Figure 12B).

***A transport model for NAD-ME C4 photosynthesis***

Overall, the combination of MC family members currently identified in plants, their characterised transport activities, and their cell-specific expression levels across multiple $C_4$ species of different subtypes, suggests a mechanism for dicarboxylate transport across the

mitochondrial membrane in NAD-ME subtype $C_4$ photosynthesis (Figure 12C). This model was first proposed by Furbank et al., before any of the transporters involved had been identified, based on the observation that isolated *P. miliaceum* bundle sheath cells and chloroplasts carry out maximal $O_2$ evolution only in the presence of a combination of aspartate, 2-OG, malate and $P_i$[93]. The model was further supported by Taniguchi and Sugiyama after the initial characterisation of DTC in a $C_4$ plant[98]. Now transporters have been identified that allow the model to be fully described. Briefly, UCP facilitates the import of aspartate in exchange for glutamate produced by mitochondrial AspAT-mediated transamination of the aspartate and 2-OG. 2-OG in turn is imported by DTC, in exchange for malate export. Malate must be reimported through DIC in exchange for $P_i$ export, driven by the $P_i$ gradient generated by $P_i$:$H^+$ symport through PIC (Figure 12C).

## *Conclusion*

Substantial progress has been made in our understanding of the metabolite transporters of the $C_4$ cycle. Although many transporters capable of catalysing the correct transport reactions required for $C_4$ photosynthesis have been characterised in $C_3$ species, few have been functionally tested in $C_4$ species and thus formal proof of their role in $C_4$ photosynthesis is lacking. Moreover, despite over 50 years passing since the first identification of the transport of $C_4$ cycle metabolites across the chloroplast inner envelope[28], there are still several important gaps in our understanding of metabolite transport in the pathway. For example, while most $C_4$ species appear to have recruited the $Na^+$-dependent BASS2 for pyruvate import into the mesophyll chloroplast[79], The transporter that facilitates the $H^+$-dependent pyruvate uptake in the Arundinelleae and Andropogoneae has yet to be discovered[74]. Similarly, bundle sheath chloroplast malate in all NADP-ME $C_4$ species import is still an open question. The best evidence to date, suggests that the majority of NADP-ME $C_4$ species utilise a multi-step valve-like mechanism that is in part facilitated by members of the DCT subfamily[41]. However, the only efficient transport activity so far observed for DCT

subfamily transporters is the exchange of four-carbon dicarboxylates (e.g. OAA, malate, succinate, fumarate, 2-OG) and four- and five-carbon amino acids (e.g. aspartate, glutamate)[24,26,108]. Significant dicarboxylate/amino acid unidirectional uptake was not been observed with DCTs from *A. thaliana* or maize[25,26], and DCT from $C_3$ spinach and $C_4$ *F. bidentis* were shown not to exchange malate and pyruvate[24]. Thus, whilst DCTs appear to be involved in the transport of dicarboxylates and amino acids across the bundle sheath chloroplast envelope during the $C_4$ cycle, they cannot facilitate the net import of carbon that is required for the $C_4$ cycle to function as a carbon-concentrating mechanism. The existence of an additional aspartate transporter (likely a cation:aspartate symporter) has been proposed to explain this[41], which is consistent with previous observations that malate-dependent pyruvate formation from wild-type maize bundle sheath cells or chloroplasts is stimulated by aspartate[42,43]. It is also plausible (and parsimonious) that there exists a separate transporter capable of either malate uptake (likely a cation:malate symporter) or simultaneous malate uptake and pyruvate efflux. In the absence of a transporter capable of carrying out malate import and pyruvate export, pyruvate export is presumably facilitated by a separate pyruvate exporter (likely a cation:pyruvate symporter). However, no such transporter is known in plants or other organisms. The identification of the missing transporters above, and their characterisation in $C_4$ plants represents one of the biggest challenges in $C_4$ biology, and is essential to realise the dream of engineering $C_4$ photosynthesis into $C_3$ plants to improve their yield.

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## *Figures*

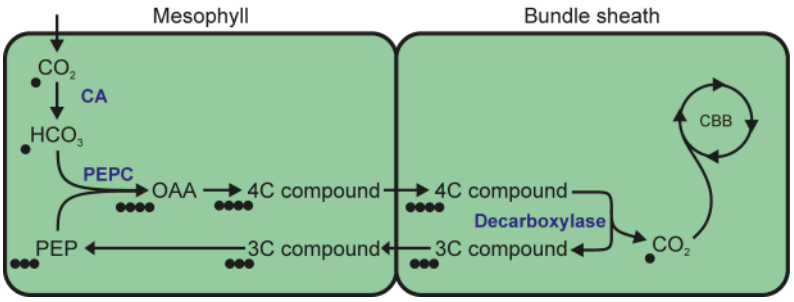


**Figure 1.** Simplified cartoon of two-cell $C_4$ photosynthesis. The 4-carbon compound may be malate or aspartate. The 3-carbon compound may be pyruvate or alanine. Metabolites are shown in black. Black circles indicate number of carbon atoms per metabolite. Enzymes are shown in blue. For abbreviations see Supplemental File 1.

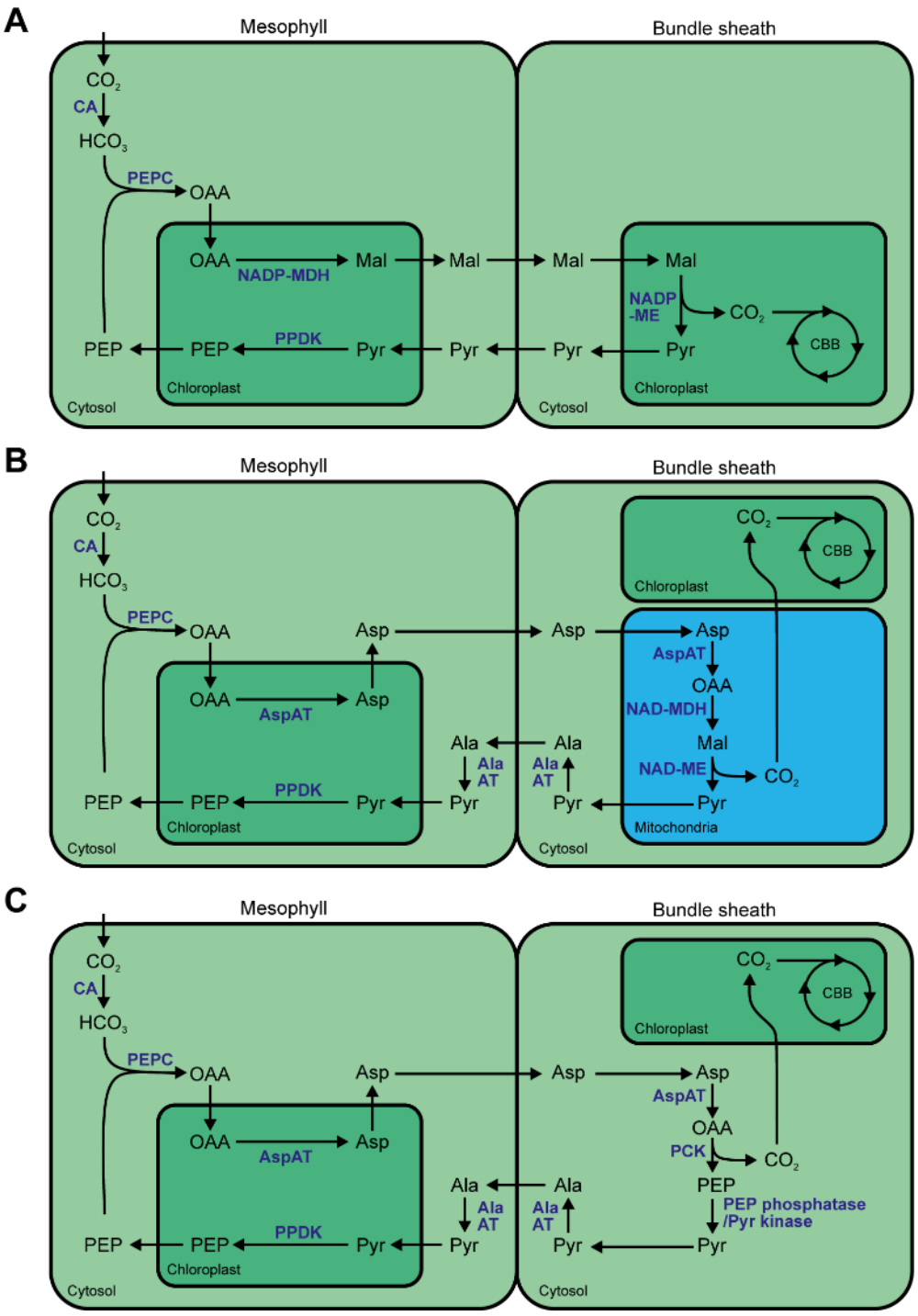


**Figure 2**: $C_4$ photosynthesis subtypes. Diagrams showing **A**) NADP-ME subtype, **B**) NAD-ME subtype, and **C**) PCK subtype $C_4$ photosynthesis. Metabolites are shown in black. Enzymes are shown in blue. For abbreviations see Supplemental File 1.

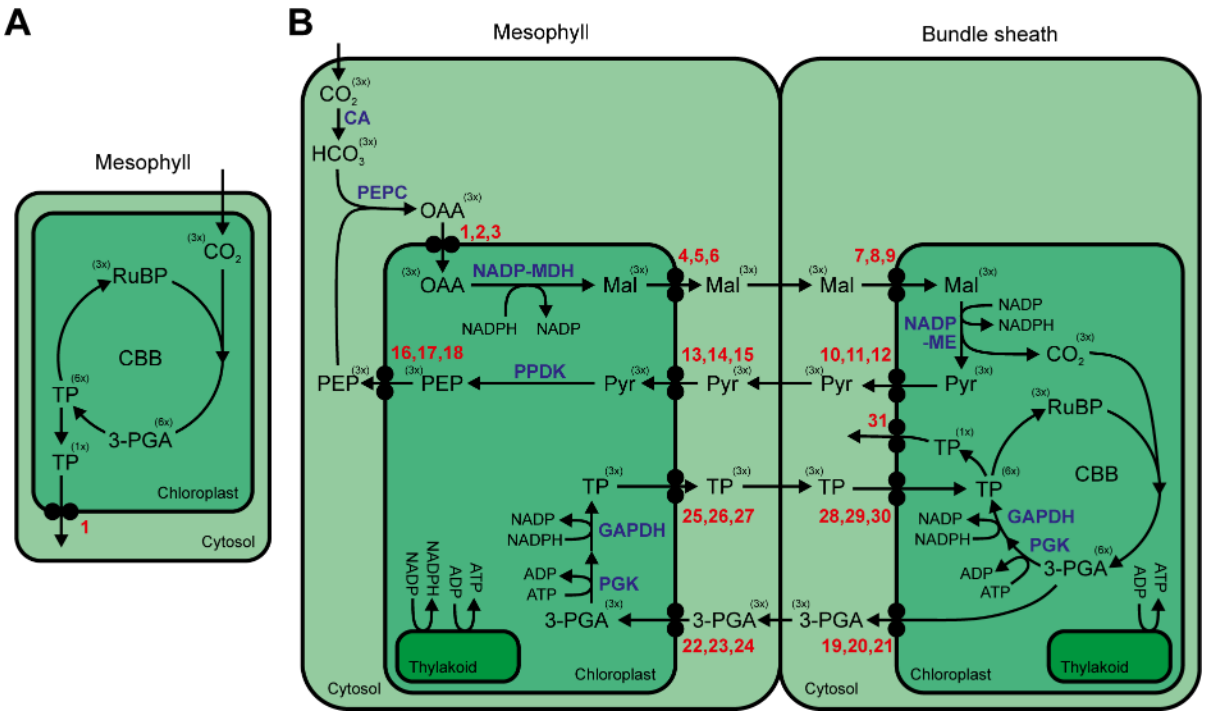


**Figure 3.** Intracellular metabolite transport in $C_3$ and $C_4$ photosynthesis. Diagrams showing minimal intracellular metabolite transport required for **A**) $C_3$ photosynthesis, and **B**) NADP-ME subtype $C_4$ photosynthesis. Metabolites are shown in black. Enzymes are shown in blue. Brackets show relative stoichiometries of each metabolite for the production and export of one molecule of TP. Counts of number of transport reaction required for the production and export of one molecule of TP are shown in red. For abbreviations see Supplemental File 1.

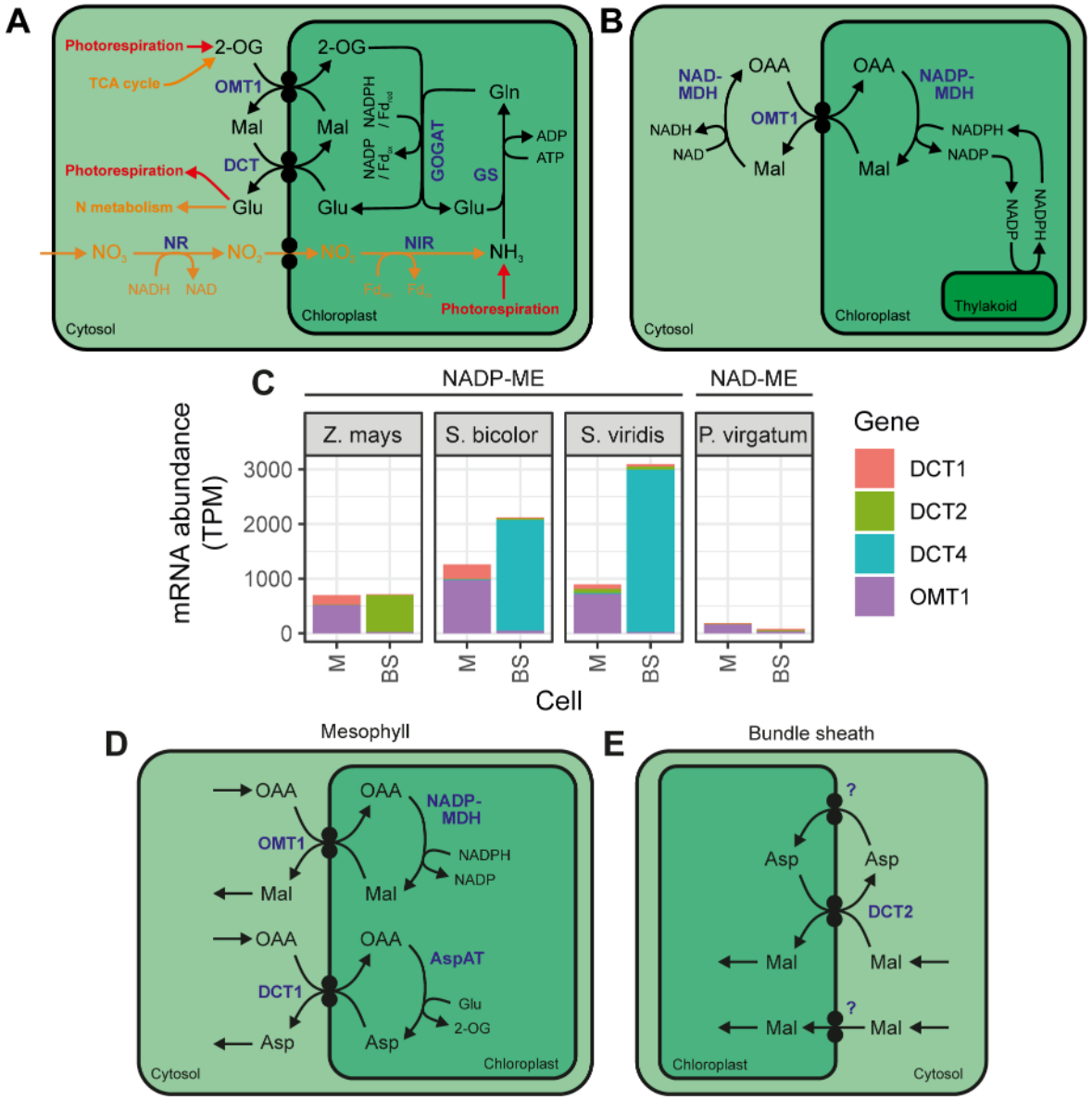


**Figure 4.** the role of DCT transporters in $C_3$ and $C_4$ plants. **A**) DCT function in nitrogen assimilation in $C_3$ plants. Entry and exit points to primary N assimilation are shown in orange, and to secondary (photorespiratory) N assimilation are shown in red. **B**) OMT1 function in redox balancing in $C_3$ plants. **C**) Cell-type specific transcript abundance of DCT family genes among NADP-ME and NAD-ME subtype $C_4$ monocots. *Z. mays* data from Chang et al. (2012)[37]. *S. bicolor* data from Emms et al. (2016)[44]. *S. viridis* data from John et al. (2014)[45]. *P. virgatum* data from Rao et al. (2016)[48]. M = mesophyll. BS = bundle sheath. TPM = transcripts per million. **D**) Function of OMT1 and DCT1 in mesophyll cell of $C_4$ plants. **E**) Hypothesised function of DCT2 in bundle sheath cell of $C_4$ plants. Metabolites are shown in black. Enzymes and transporters are shown in blue. For abbreviations see Supplemental File 1.

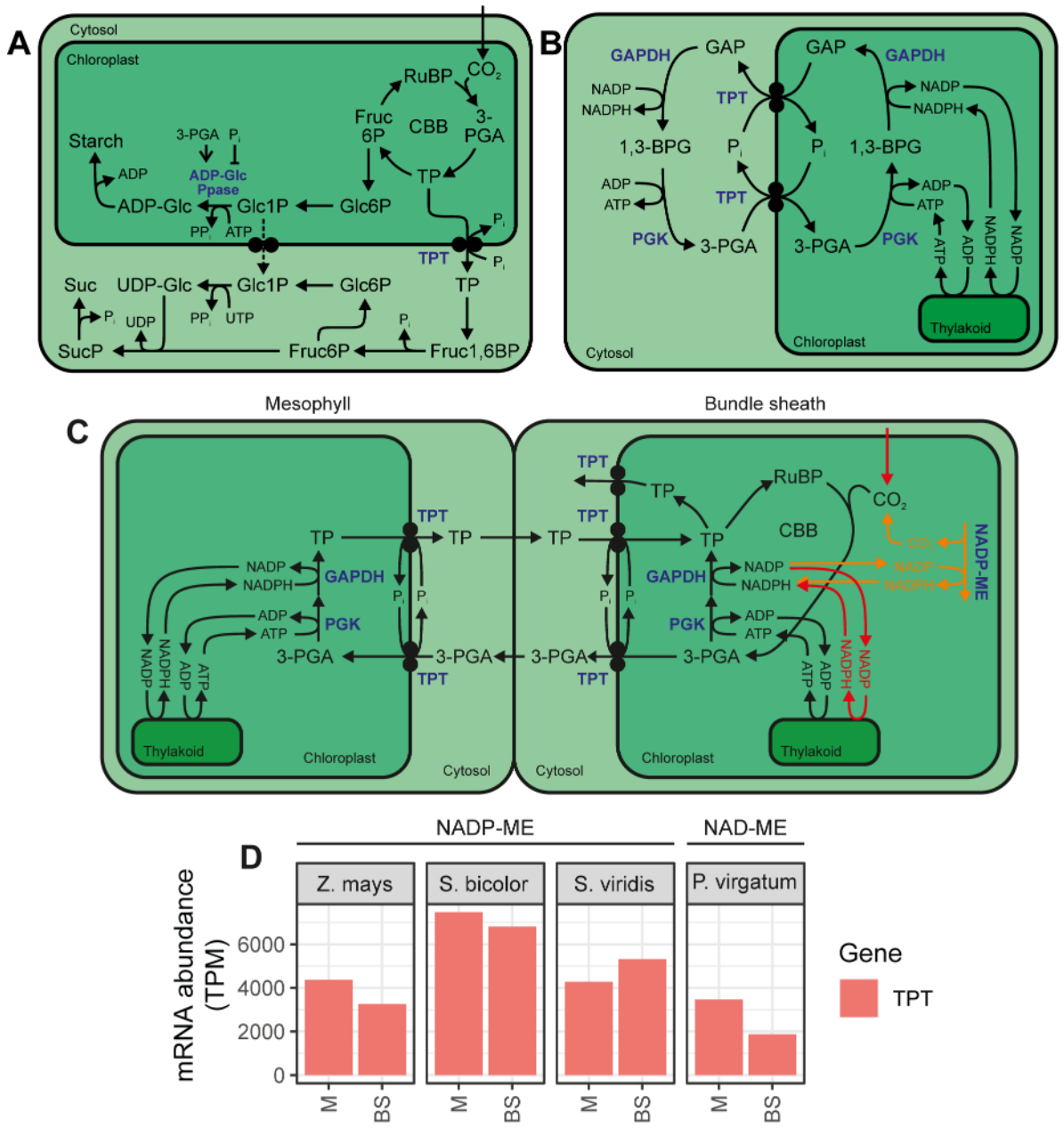


**Figure 5.** The role of TPT in $C_3$ and $C_4$ plants. **A**) TPT function in photosynthetic TP export and coordination of starch and sucrose biosynthesis in $C_3$ plants. **B**) TPT function in exporting reducing equivalents and ATP from the chloroplast in $C_3$ plants. **C**) Functions of TPT in $C_4$ plants. NADP-ME subtype-only parts of pathway are shown in orange. NAD-ME subtype-only parts of pathway are shown in red. **D**) Cell-type specific transcript abundance of TPT genes among NADP-ME and NAD-ME subtype $C_4$ monocots. Data sources described in Figure 4. M = mesophyll. BS = bundle sheath. TPM = transcripts per million. Metabolites are shown in black. Enzymes and transporters are shown in blue. For abbreviations see Supplemental File 1.

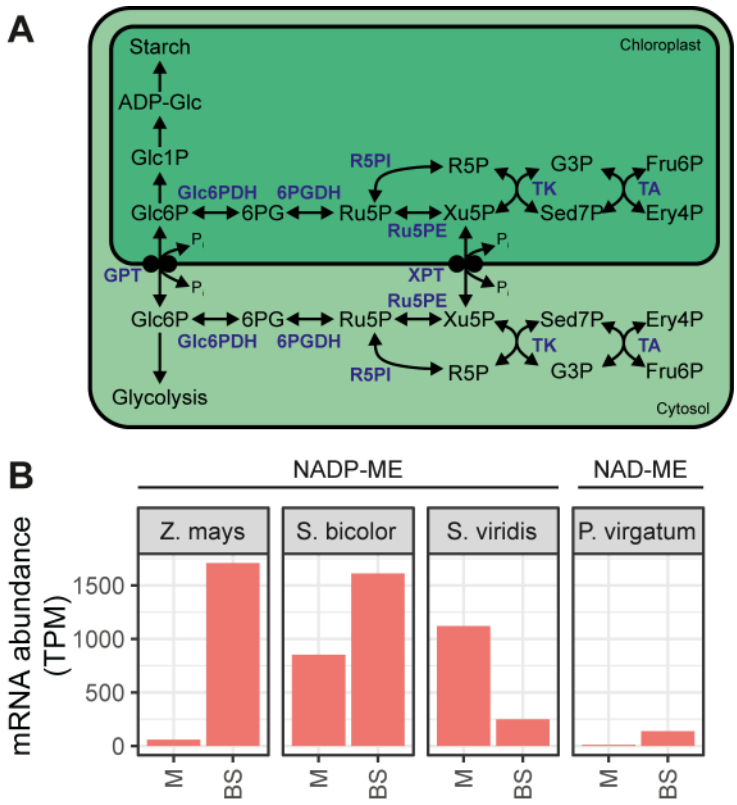


**Figure 6.** GPT and XPT in $C_3$ plants and $C_4$ plants. **A)** The role of GTP and XPT in $C_3$ plants. **B)** Cell-type specific transcript abundance of GPT among NADP-ME and NAD-ME subtype $C_4$ monocots. Data sources described in Figure 4. M = mesophyll. BS = bundle sheath. TPM = transcripts per million. Metabolites are shown in black. Enzymes and transporters are shown in blue. For abbreviations see Supplemental File 1.

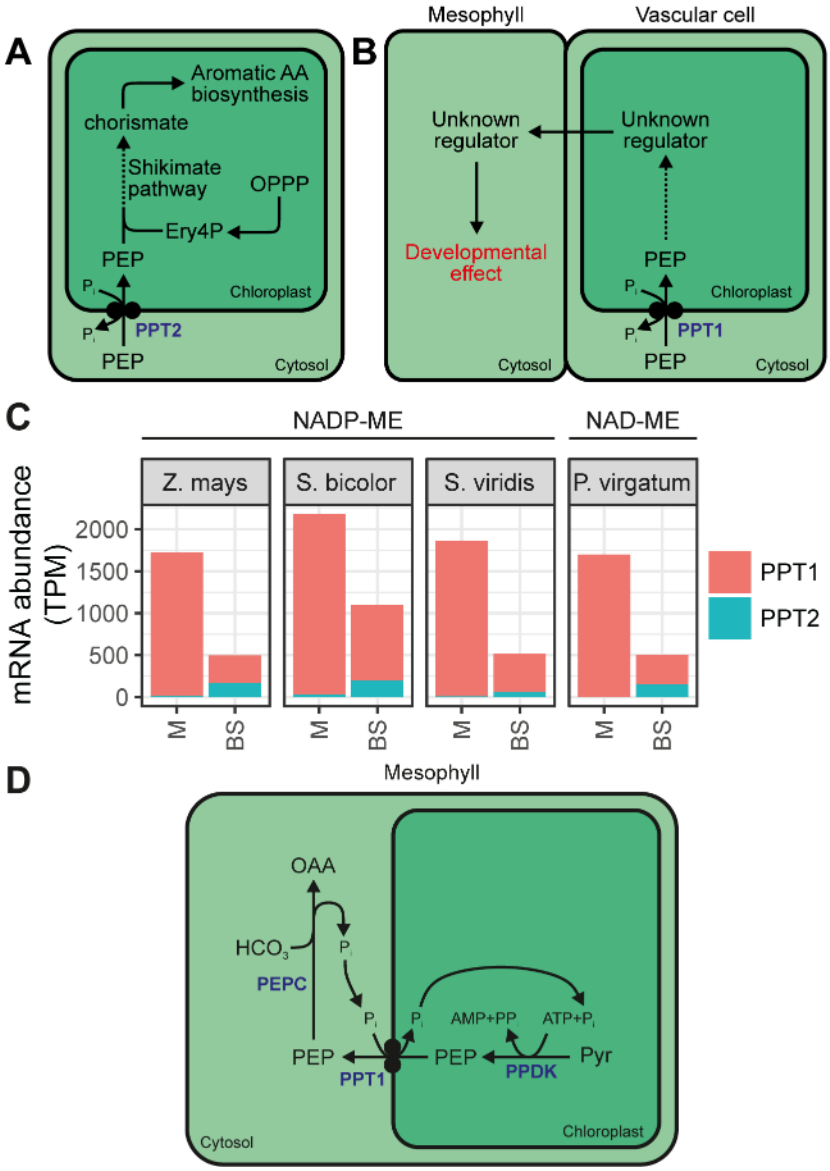


**Figure 7.** The role of PPT in $C_3$ and $C_4$ plants. **A**) PPT2 function in chloroplastic PEP import for the shikimate pathway in $C_3$ plants. **B**) PPT1 has a developmental function, possibly by phenolic compounds. **C**) Cell-type specific transcript abundance of PPT genes among NADP-ME and NAD-ME subtype $C_4$ monocots. Data sources described in Figure 4. **D**) Function of PPT in $C_4$ plants. Metabolites and pathways are shown in black. Transporters are shown in blue. For abbreviations see Supplemental File 1.

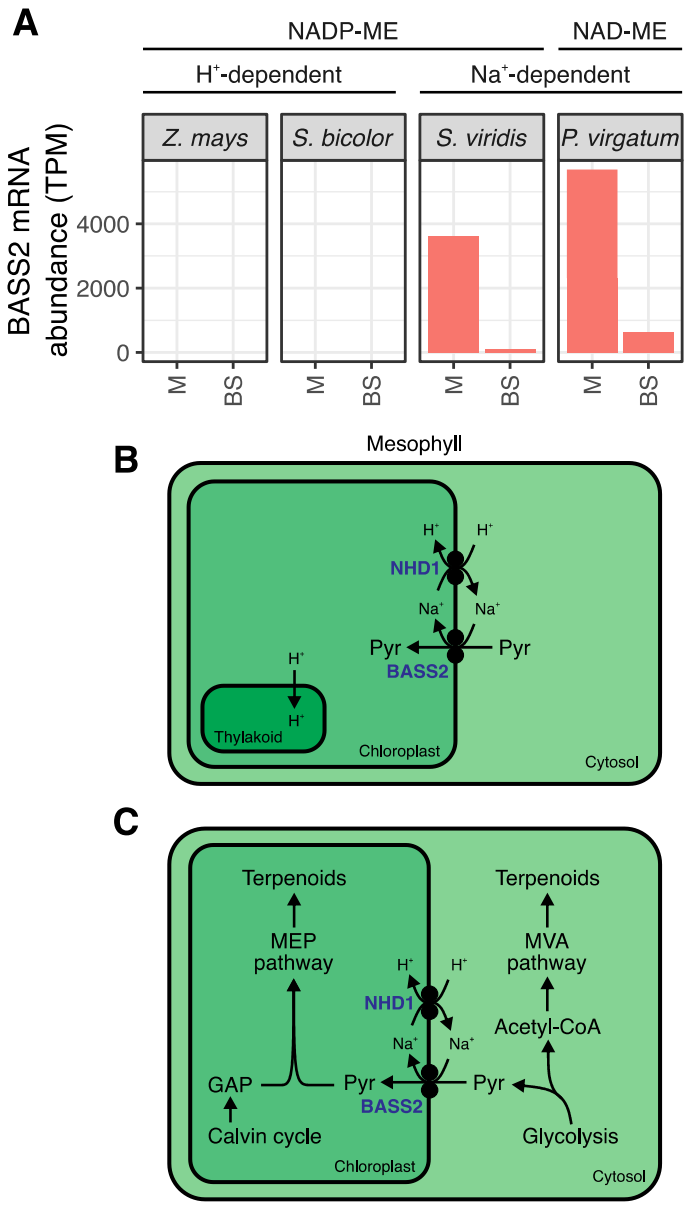


**Figure 8**. The role of BASS2 in $C_3$ and $C_4$ plants. **A**) Cell-type specific transcript abundance of BASS2 gene among NADP-ME and NAD-ME subtype $C_4$ monocots. $H^+$- or $Na^+$-dependence of mesophyll cell pyruvate uptake is also indicated. M = mesophyll. BS = bundle sheath. TPM = transcripts per million. **B**) Function of BASS2 in $C_4$ plants. **C**) Function of BASS2 in $C_3$ plants. Metabolites are shown in black. Enzymes and transporters are shown in blue. For abbreviations see Supplemental File 1.

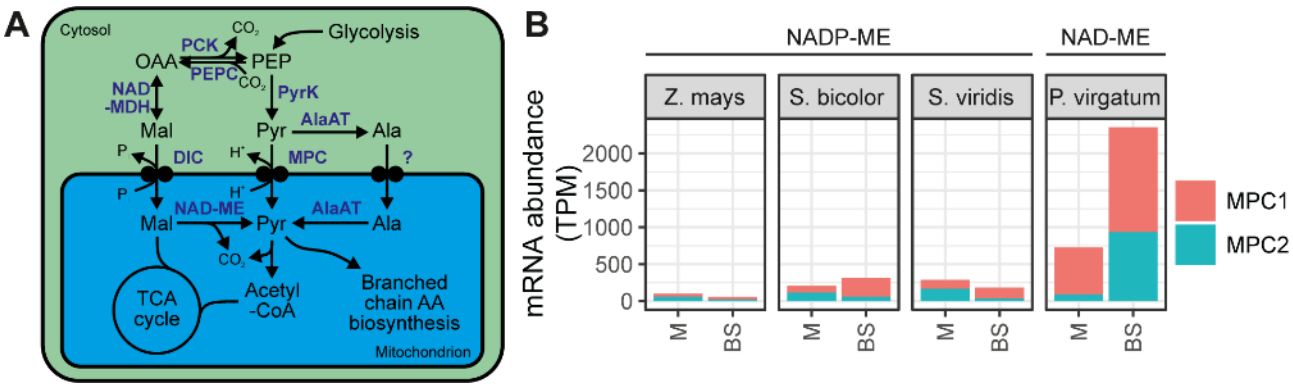


**Figure 9.** Function of MPC in $C_3$ and $C_4$ plants. **A**) The function of MPC in $C_3$ plants. **B**) Cell-type specific transcript abundance of BASS2 gene among NADP-ME and NAD-ME subtype $C_4$ monocots. Metabolites and pathways are shown in black. Enzymes and transporters are shown in blue. For abbreviations see Supplemental File 1.

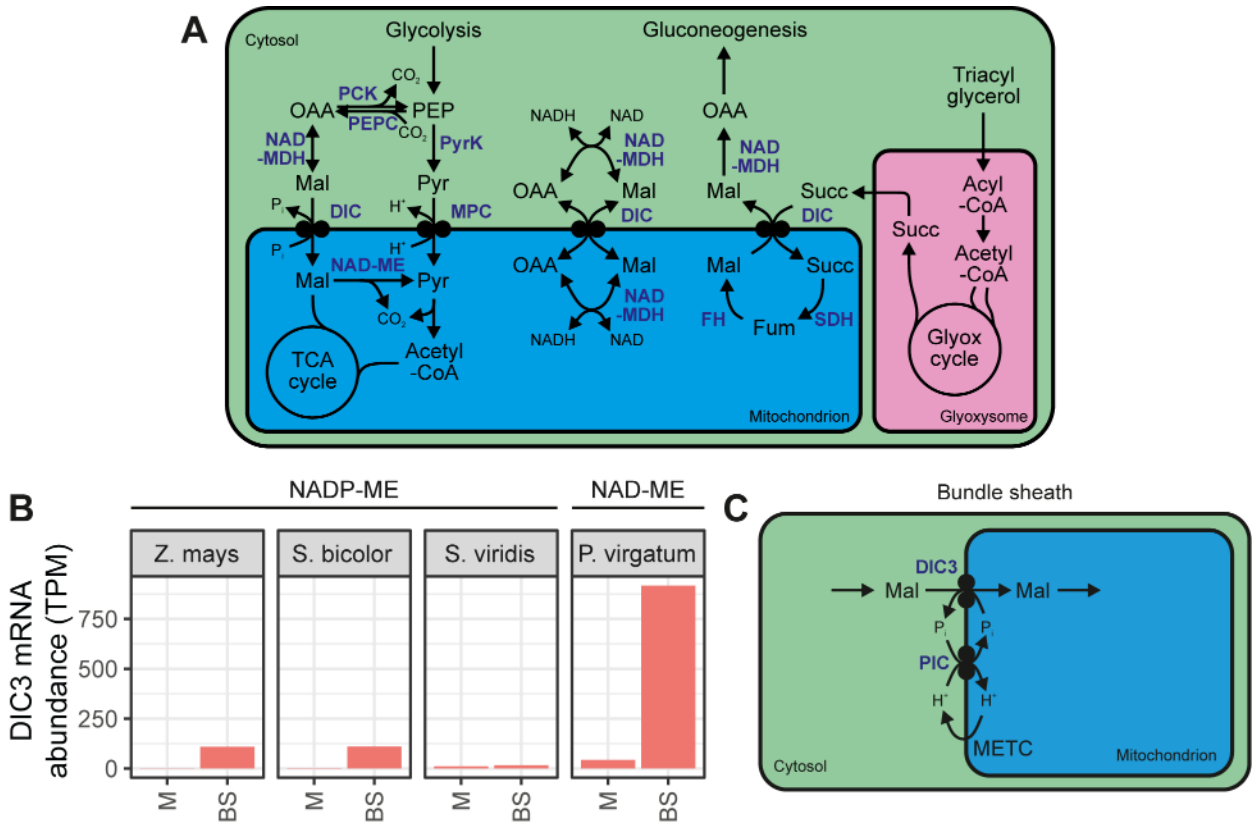


**Figure 10.** Possible functions of DIC in $C_3$ and $C_4$ plants. **A**) Left: Anaplerotic provision of malate to TCA cycle. Centre: Exchange of reducing equivalents between cytosol and mitochondria. Right: Mitochondrial malate/succinate exchange in conjunction with glyoxylate cycle and gluconeogenesis. **B**) Cell-type specific transcript abundance of DIC genes among NADP-ME and NAD-ME subtype $C_4$ monocots. **C**) Function of DIC in $C_4$ plants. METC = mitochondrial electron transport chain. Metabolites and pathways are shown in black. Enzymes and transporters are shown in blue. For abbreviations see Supplemental File 1.

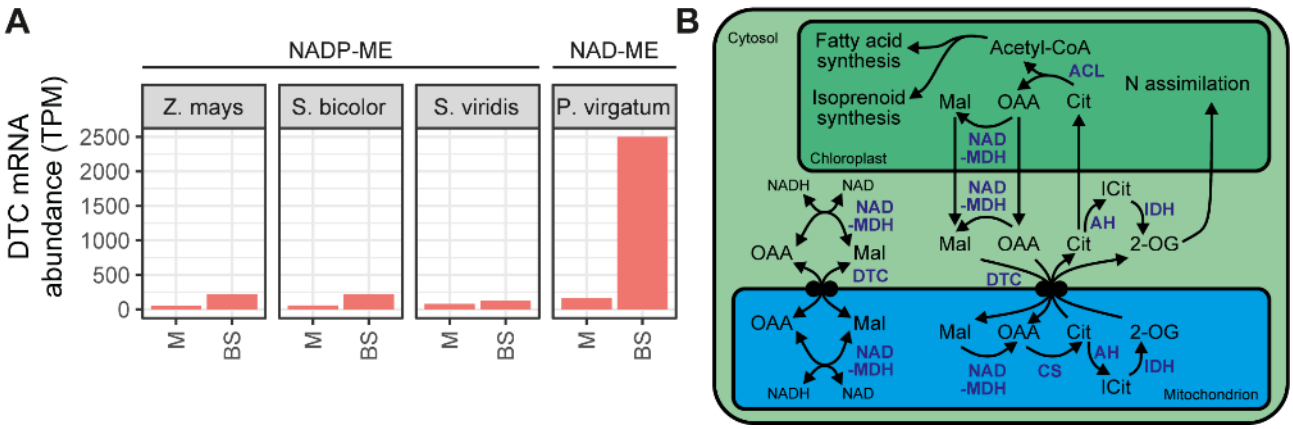


**Figure 11**. The role of DTC in $C_3$ and $C_4$ plants. **A**) Cell-type specific transcript abundance of DTC genes among NADP-ME and NAD-ME subtype $C_4$ monocots. M = mesophyll. BS = bundle sheath. TPM = transcripts per million. **B**) Possible functions of DTC in $C_3$ plants. Left: Exchange of reducing equivalents between cytosol and mitochondria. Right: Exchange of malate and/or oxaloacetate for citrate and 2-OG for chloroplastic fatty acid/isoprenoid synthesis and nitrogen assimilation respectively. Metabolites and pathways are shown in black. Enzymes and transporters are shown in blue. For abbreviations see Supplemental File 1.

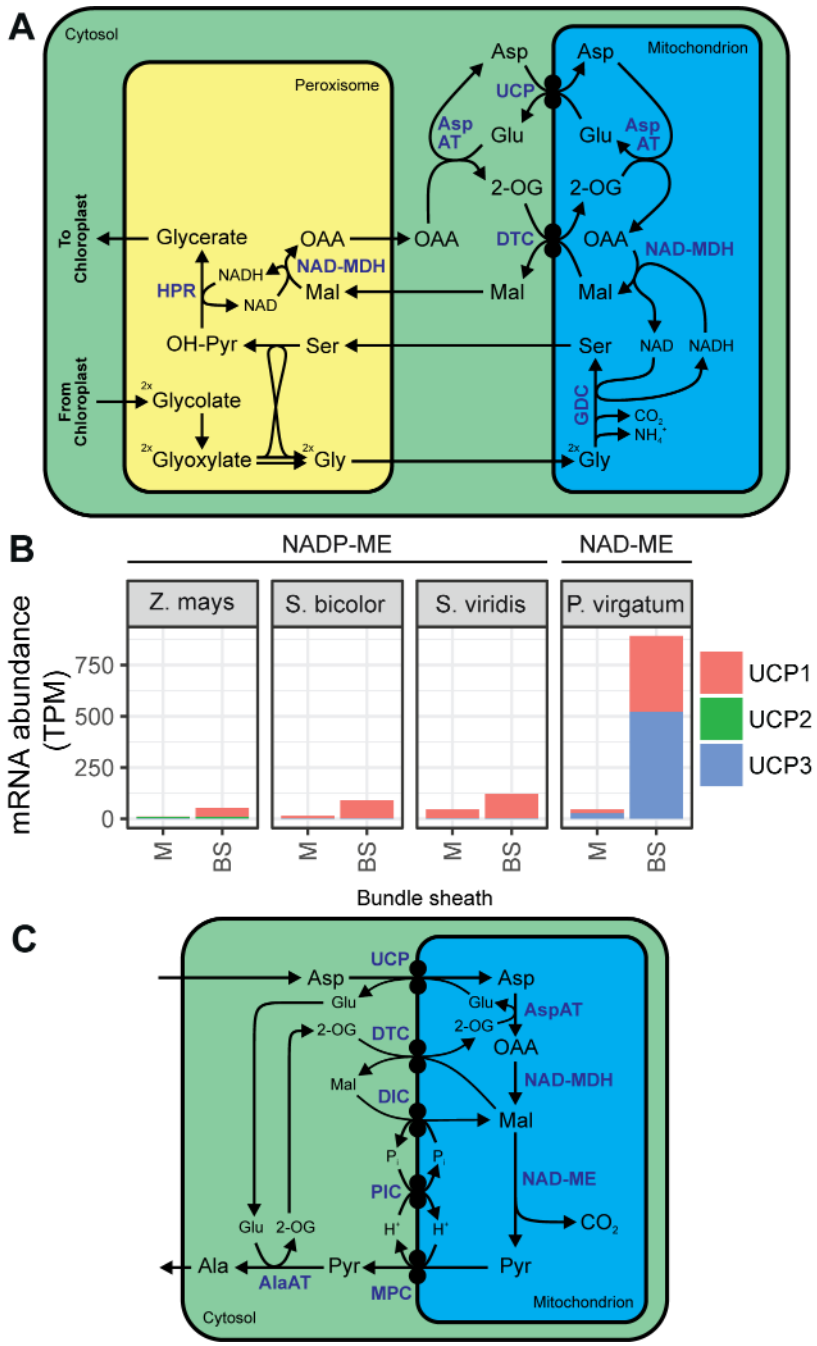


**Figure 12**. The role of UCP in $C_3$ and $C_4$ plants. **A**) The role of UCP transporters in respiration and photorespiration in $C_3$ plants. **B**) Cell-type specific transcript abundance of UCP genes among NADP-ME and NAD-ME subtype $C_4$ monocots. M = mesophyll. BS = bundle sheath. TPM = transcripts per million. **C**) Hypothesis for Metabolite transport at the mitochondrial envelope to support NAD-ME subtype $C_4$ plants. Metabolites are shown in black. Enzymes and transporters are shown in blue. For abbreviations see Supplemental File 1

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