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Understanding metabolite transport and metabolism in C_4 plants through RNA-seq

Urte Schlüter[1], Alisandra K Denton[2] and Andrea Bräutigam[1,3]

RNA-seq, the measurement of steady-state RNA levels by next generation sequencing, has enabled quantitative transcriptome analyses of complex traits in many species without requiring the parallel sequencing of their genomes. The complex trait of C_4 photosynthesis, which increases photosynthetic efficiency via a biochemical pump that concentrates CO_2 around RubisCO, has evolved convergently multiple times. Due to these interesting properties, C_4 photosynthesis has been analyzed in a series of comparative RNA-seq projects. These projects compared both species with and without the C_4 trait and different tissues or organs within a C_4 plant. The RNA-seq studies were evaluated by comparing to earlier single gene studies. The studies confirmed the marked changes expected for C_4 signature genes, but also revealed numerous new players in C_4 metabolism showing that the C_4 cycle is more complex than previously thought, and suggesting modes of integration into the underlying C_3 metabolism.

Addresses
[1] Institute of Plant Biochemistry and Cluster of Excellence on Plant Science (CEPLAS), Heinrich-Heine-University, Universitätsstrasse 1, D-40225 Düsseldorf, Germany
[2] Institute for Biology I, RWTH Aachen University, Worringer Weg 3, 52074 Aachen, Germany

Corresponding author: Bräutigam, Andrea (braeutigam@ipk-gatersleben.de)
[3] Present address: Network Analysis and Modeling Group, IPK Gatersleben, Corrensstrasse 3, D-06466 Stadt Seeland, Germany.

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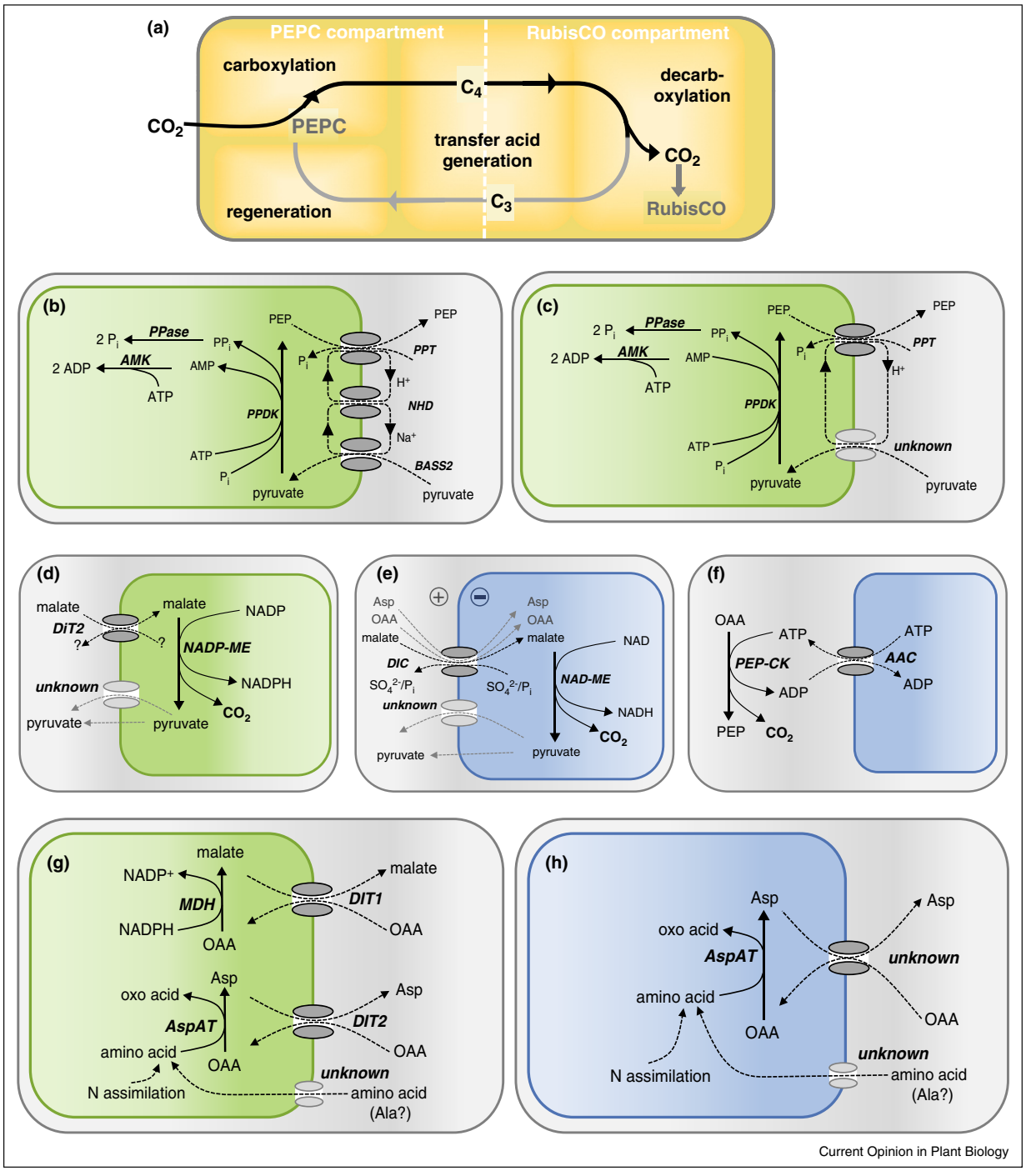
Introduction

The complex trait of C_4 photosynthesis requires numerous intra-cellular and inter-cellular transport processes [1,2]. Species which have evolved the trait supercharge photosynthesis by pre-fixing CO_2 through phospho*enol*-pyruvate carboxylase (PEPC) in the mesophyll, transport of C_4 metabolites to the bundle sheath, and decarboxylating and enriching CO_2 at the site of RubisCO. The higher affinity of PEPC for CO_2 compared to RubisCO lowers the CO_2 compensation point of the plants. This allows C_4 plants to thrive in environments that are carbon limited, that is, environments with high light, high temperature, and low or seasonal water availability [3].

By comparing sister species with and without the trait on a global scale, RNA-seq can identify trait-related differences in transcript abundance. The global perspective, which tests all genes at the same time, identifies both changes that are expected based on previously published data (i.e. enzyme assays or Western Blots; summarized in [4]) and changes that are not anticipated. Unlike the targeted approaches that existed before, RNA-seq is not limited to measuring abundance of known or expected transcripts. The downside is that any changes happening after transcript abundance such as translation efficiency, protein stability and modification are invisible to the approach. The initial comparative RNA-seq paper found that the abundance of all known C_4 cycle transcripts except malate dehydrogenase is indeed increased in the C_4 plant compared to a closely related C_3 plant [5••]. This indicates that the complex trait C_4 photosynthesis is traceable at the transcript abundance level [5••]. After the initial comparison in the genus *Cleomaceae* [5••], the C_4 trait has also been traced in the *Flaveriaceae* [6,7], the (X = 9) *Panicoideae* [8], and in comparison of grass species with wider phylogenetic spacing, rice (*Ehrhartoideae*) vs *Echinocloa* (X = 9 *Panicoideae*) [9•] and rice (*Ehrhartoideae*) vs maize (*Andropogonae*) [10].

C_4 photosynthesis is a complex trait which consists of changes in cellular and leaf tissue architecture, changes in regulation, and changes in metabolism. The metabolic aspect of the trait can be deconstructed in the C_4 cycle itself consisting of carboxylation, transfer acid generation, decarboxylation and regeneration modules and their integration in the underlying leaf metabolism which presumably resembles that of non-C_4 plants (Figure 1, Table 1). Originally, three types of C_4 cycles were recognized, the NADP-malic enzyme (NADP-ME), the NAD-malic enzyme (NAD-ME) and the phospho*enol*pyruvate (PEP) carboxykinase (PEP-CK) type [4] and they were named according to the presence of decarboxylation enzymes [4]. This simplistic view was theoretically questioned [11] at the same time as multiple lines of evidence showed a more complex cycle than previously envisioned [12–14]. As the C_4 cycle changes with developmental status [13] and possibly also with biotic and abiotic conditions [11], it seems prudent to no longer focus on historical prototypes but to describe the cycle as a series

Figure 1



C_4 metabolism is carried out by a combination of carboxylation, transfer acid generation, decarboxylation and regeneration modules. **(a)** Contribution of different modules to C_4 metabolism; **(b, c)** regeneration of PEP by the plastidic enzyme PPDK in the mesophyll, transport of the reaction product is performed by PPT, the import of pyruvate can be mediated by sodium coupled BASS2/NHD system (b) or by an unknown pyruvate transporter in the *Andropogonae* (c); **(d)–(f)** decarboxylation of the transfer metabolite in the bundle sheath, NADP-ME decarboxylation (d) is localized in the chloroplast with DiT2 a likely malate importer candidate in maize and the pyruvate exporter unknown (d); NAD-ME decarboxylation takes place in the mitochondria, import of malate is predicted to be carried out by the DIC translocator, the pyruvate exporter is unknown (e), Decarboxylation by PEP-CK in the cytosol is likely to depend on transport of ATP from the mitochondria by AAC; **(g, h)** transfer metabolite generation in the mesophyll. Malate and aspartate are produced in the plastid of the mesophyll especially in species with NADP-ME decarboxylation, their export is mediated by DiT1 and DiT2 respectively, aspartate can also be synthesized in the mitochondria, but a cooperating transporter is still unknown. Transport of aspartate out of the plastid would require recycling of the amino group, this could be supplied directly from N assimilation or by import of amino acid via an unknown mechanism. For abbreviations, see text.

Table 1

Prototypical C_4 cycle genes; all data given as reads per million; *P. maximum* values taken from Bräutigam *et al.* (2014), *Z. mays* whole leaf values taken from Sekhon *et al.* (2011), leaf VT_13, *G. gynandra* values remapped from Kulahoglu *et al.* (2014), leaf stage 4, *F. trinervia* values averaged from Mallmann *et al.* (2014), *Z. mays* mesopyll and bundle sheath (BS) data from Chang *et al.* (2012); action: E = enzyme; T = transporter; compartment: cp = chloroplast, cy = cytosol, mi = mitochondria

Module	Gene name	Compartment	Action	Arabidopsis ID[b]	Reaction	*P. maximum*	*G. gynandra*	*F. trinervia*	*Z. mays* whole leaf	*Z. mays* mesophyll	*Z. mays* BS
Regeneration module	PPDK	cp	E	AT4G15530	Pyruvate + 2 ATP + P → PEP + AMP + PP	13,380	20,941	25,638	11,092	35,421	7810
	AMK	cp	E	At5g47840	AMP + ATP → 2 ADP	986	2328	1916	527	4739	1018
	Ppase	cp	E	At5g09650	PP → 2 P	451	1828	1138	371	579	286
	BASS2	cp	T	At2g26900	Pyruvate (out) + Na^+ (out) → pyruvate (in) + Na^+ (in)	2797	4298	5790	nd	nd	nd
	NHD	cp	T	At3g19490	H^+ (out) + Na^+ (in) → H^+ (in) + Na^+ (out)	838	1081	1592	5	7	3
	PPT	cp	T	At5g33320	PEP (in) + H^+ (in) + P (out) → PEP (out) + H^+ (out) + P (in)	405	1633	2225	562	1498	243
	Proton pyruvate transporter	cp	T	Unknown	pyruvate (out) + H^+ (out) → pyruvate (in) + H^+ (in)	Not needed	Not needed	Not needed	?	?	?
Carboxylation	PEPC	cy	E	At2g42600	PEP + HCO_3^- → OAA + P	18,393	19,879	15,712	5053	9485	432
	cytCA	cy	E	Multiple solutions	CO_2 + H_2O → HCO_3^- + OH^-	28,838	3081	10,493	2990	6692	281
Decarboxylation	NADP-ME	cp	E	At1g79750	Malate + NADP → pyruvate +NADPH +CO_2	1120.5[a]	1	4649	1334	87	12,192
	DiT2/DCT	cp	T	At5g64280	Malate (out) + X → malate (in) + X	Not needed	Not needed	212	149	7	545
	pyruvate exporter	cp	T	Unknown	Pyruvate (in) + X → pyruvate (out) + X	Not needed	Not needed	?	?	?	?
	NAD-ME	mi	E	At2g13560	Malate + NAD → pyruvate +NADH +CO_2	177.5[a]	2624	172[a]	14	7	29
	DIC	mi	T	At2g22500	Malate (out) + P or SO_4^- (in) → malate (in) + P or SO_4^- (out)	455	577	6	29	2	147
	pyruvate exporter	mi	T	Unknown	Pyruvate (in) + X → pyruvate (out) + X	?	?	Not needed	Not needed	Not needed	Not needed
	PEP-CK	cy	E	At4g37870	OAA + ATP → PEP + ADP + CO_2	8819	106	42	1364	21	7801
	AAC	mi	T	At3g08580	ATP (in) + ADP (out) → ATP (out) + ADP (in), stoichiometry not clear for P	461	578	487	83	35	115
Transfer acid generation	plAspAT	cp	E	At4g31990	OAA + aminoacid → Asp + ketoacid	49	151	1476	436	1797	51
	plMDH	cp	E	At5g58330	OAA + NADPH → malate + NADP	632	478	6165	702	2621	95
	DiT1/OMT	cp	T	AT5G12860	OAA (out) + malate (in) → malate (out) + OAA (in)	203	409	744	310	476	22
	DiT2/DCT	cp	T	AT5G64280	OAA (out) + Asp (in) → Asp (out) + OAA (in)	33	153	212	149	7	545
	mAspAT	mi	E	At2g30970	OAA + aminoacid → Asp + ketoacid	16	3885	246	23	13	8
	mMDH	mi	E	AT1G53240	OAA + NADH → malate + NAD	164	721	185	77	28	104
	cytAspAT	cy	E	At1g62800	OAA + aminoacid → Asp + ketoacid	1273	5	44	24	19	91
	cytMDH	cy	E	AT1G04410	OAA + NADH → malate + NAD	735	4	5	311	463	1083
	AlaAT	?	E	At1g17290	Pyruvate + amino acid → alanine + ketoacid	3000	2538	2628	997	349	222

Table 1 (*Continued*)

Module	Gene name	Compartment	Action	Arabidopsis ID[b]	Reaction	*P. maximum*	*G. gynandra*	*F. trinervia*	*Z. mays* whole leaf	*Z. mays* mesophyll	*Z. mays* BS
Integration	TPT	cp	T	At5g46110	TP (in) + 3-PGA (out) → TP (out) + 3-PGA (in)	2757	6022	3555	730	2069	1551
	PGK	cp	E	AT3G12780	3-PGA + ATP → 2,3 bisphosphoglycerate + ADP	1568	2698	2160	644	2630	774
	GAP-DH	cp	E	AT1G42970	2,3 bisphosphoglycerate + NADPH → TP + NADP	6896	4520	5930	446	303	509
	PDH-kinase	mi	E	AT3G06483	PDH protein + ATP → PDH protein-phosphorylated + ATP	920	393	118	674	314	319

[a] May be mismapped to wrong ME during cross-species mapping.
[b] Representative gene, not always the best blast hit.

of modules which can be linked differently in different plants. The reactions involved in the C_4 cycle are characterized by a high degree of compartmentation. In the majority of plants, the carboxylation and decarboxylation modules are separated into different cell types, the mesophyll and bundle sheath, but in a variety of *Chenopdiaceae* and Hydrilla they also occur in the same cell [15]. Many enzymes involved in the C_4 cycle reside in organelles rather than in the cytosol [4]. Their localization necessitates the presence of transport proteins which connect the reactions of the C_4 cycle to the cytosol [16]. In turn, the cytosols of the PEPC containing mesophyll tissue and the RubisCO containing bundle sheath tissue share a symplastic connection [17–20]. Molecular identification of the transport proteins lags behind identification of the enzymes. The necessary transport functions for each module will be highlighted in this review.

Modules of the C_4 cycle

Regeneration module

The regeneration module produces the CO_2 acceptor, phospho*enol*pyruvate (PEP), from pyruvate and phosphate. It is invariably localized to the chloroplasts with the pyruvate phosphate dikinase (also known as pyruvate orthophosphate dikinase) as the central enzyme [4,21–23]. It produces PEP, pyrophosphate (PP) and adenosine monophosphosphate (AMP) from pyruvate, phosphate and ATP. The module requires the action of pyrophosphorylase (PPase) and AMP kinase (AMK) to drive the reaction and funnel PP and AMP back into metabolism as phosphate and ADP [23]. The module is only 80% specific to the mesophyll in *Zea mays* [24•] and *Sorghum bicolor* [25] based on transcript abundances (Table 1, data extracted from [24•]), but also controlled at the post-transcriptional level [26]. Two prototypes of the module are currently known (Figure 1b and c). Both use the same enzymes but different transport proteins. All species which import pyruvate in a sodium dependent manner, that is all C4 species tested so far with the exception of the *Andropogonae* [27] likely use the BASS2/NHD/PPT transport system (Figure 1b). BASS2 was identified as the probable pyruvate transporter based on expression patterns similar to other C_4 genes, that is, highly expressed in C_4 species and differentially expressed between C_3 and C_4 sister species [5••,7] and subsequently biochemically characterized [28••]. It indeed transports pyruvate in exchange for sodium, is specific for the sodium ion over lithium, is a highly abundant protein in the sodium dependent pyruvate transporting C_4 species and, if mutated in a C_3 plant, produces a pyruvate transport related phenotype [28••]. It acts in concert with a sodium protein exchanger (NHD) which exchanges the sodium for protons. The proton is transported back over the membrane with the export of PEP and import of phosphate. The transport across the chloroplast envelope thus involves three transport proteins which catalyze a net flux of pyruvate and phosphate in and PEP out with sodium

and protons cycling [28••]. The transcripts of all three transporters are highly abundant in C_4 plants and differentially expressed compared to sister C_3 species (Table 1) [5••,7,8].

The C_4 *Andropogonae* such as sorghum or maize are known to exchange pyruvate for protons directly [27] and consequently, neither BASS2 nor NHD can be detected as highly abundant in maize (Table 1) [25]. Instead of the conjunct action of BASS and NHD, the *Andropogonae* have an alternative transport protein in mesophyll chloroplasts which, to date, has not been characterized at the molecular level (Figure 1c, Table 1). Candidates for the chloroplast pyruvate importer in maize were suggested based on proteomic comparison of maize and pea chloroplast envelopes: mesophyll envelope proteins (Mep) Mep1-4 (Table 2) [16]. A pyruvate transporter is expected to be highly abundant in maize mesophyll cells. Of the candidates originally proposed, Mep1 and to a lesser degree Mep3a, fit the above criterium. In *A. thaliana*, the transporter orthologous to Mep1 carries glycolate and glycerate during photorespiration [29] and is named PLGG1.

Carboxylation module

The product of the regeneration module, PEP, is carboxylated by PEPC in the cytosol of the cell using HCO_3^-. PEPC is always highly upregulated in C_4 species (Table 1) [5••,7,8,9•,30]. The HCO_3^- for PEPC is produced through the action of a cytosolic carbonic anhydrase (CA). Abundant cytosolic CA activity can be achieved by upregulating the cytosolic CA [5••] or by retargeting the already abundant plastidic CA [31,32]. Its mesophyll specificity is regulated post-transcriptionally [36]. The importance of the enzyme apparently varies among species. In *Flaveria bidentis*, reduction of CA drastically reduced fitness under greenhouse conditions [33] but in maize, reduction of CA only has minor effects [34]. The module is specific to the mesophyll (Table 1). No transport proteins are required for this module.

Decarboxylation module

Three alternatives are realized for decarboxylating the C_4 acid (Figure 1d–f). Based on the available global datasets, NAD-ME based and NADP-ME based decarboxylation appear to exclude each other while either can be supplemented with PEP-CK based decarboxylation [5••,6–8,9•,10]. NADP-ME based decarboxylation occurs in the chloroplasts and requires the import of malate and the export of pyruvate (Figure 1d). Transcripts of NADP-ME are reliably of higher abundance in *F. bidentis* (Table 1) [6,7] as well as in *Z. mays*, *Setaria viridis*, and *Echinochloa glabrescens* [8,9•,10]. The transport protein catalyzing the import of malate and/or the export of pyruvate is unknown at the molecular level (Table 1). The dicarboxylate transporter DiT2 (alternate name DCT) was considered a candidate transporter for malate in maize [35], however, the *Flaveria trinervia* DiT2 is unable to catalyze the reaction based on *in vitro* analysis of the transport substrates [36]. DiT2 is moderately upregulated in conjunction with NADP-ME [6–8] and DiT2 is strongly expressed in maize bundle sheath cells (Table 1) and *S. bicolor* bundle sheath cells [25]. Mutant analysis of maize showed symptoms consistent with a role in malate import in the C_4 cycle [37••] raising the question whether the original assay was unsuitable for detecting the exchange [36] or whether Flaveria and maize may use different transporters (Figure 1d). The molecular identity of the pyruvate exporter is currently unknown but it cannot be excluded that pyruvate may rely on diffusion to pass the chloroplast membrane in its electroneutral form, pyruvic acid [38,39], especially given the high concentrations of pyruvate generated by NADP-ME. The module is specific to the bundle sheath (Table 1).

The NAD-ME based decarboxylation module is built around the mitochondrial NAD-ME which is highly upregulated in the C_4 species using this type of decarboxylation (Figure 1e) [5••,8,40]. Its high expression is accompanied by the expression of a dicarboxylate carrier, DIC (Table 1) [5••,8]. Among the mitochondrial carriers that accept malate or OAA, DIC is unique as it catalyzes net carbon import as malate/phosphate or malate/sulfate exchange [41]. In case of phosphate exchange, the electrical field is capable of driving the exchange as malate is negatively charged twice and phosphate is negatively charged three times. The resulting cytosolic phosphate

Table 2

Candidate transporters for reaction uncharacterized at the molecular level; all data given as reads per million; *P. maximum* values taken from Bräutigam *et al.* (2014), *Z. mays* whole leaf values taken from Sekhon *et al.* (2011), leaf VT_13, *G. gynandra* values remapped from Kulahoglu *et al.* (2014), leaf stage 4, *F. trinervia* values averaged from Mallmann *et al.* (2014), *Z. mays* mesopyll and bundle sheath (BS) data from Chang *et al.* (2012); action: E = enzyme; T = transporter

Original name	AGI	Maize ID	*P. maximum*	*G. gynandra*	*F. trinervia*	*Z. mays* whole leaf	*Z. mays* M	*Z. mays* BS
Mep1	At1g32080	GRMZM5G873519	103	172	128	345	340	535
Mep2	At5g23890	GRMZM2G077222	38	200	169	32	0	0
Mep3	At5g12470	GRMZM2G138258	22	185	1072	498	20	2734
Mep4	At5g12470	GRMZM2G305851	3			33	113	9

or sulfate needs to be reimported, likely as proton symport [8]. If instead the C_4 acid is imported into the bundle sheath mitochondria as aspartate, the import protein remains unknown as the DICs of Arabidopsis do not accept amino acids [41].

The decarboxylation yields mitochondrial pyruvate which has to be exported to the cytosol. The pyruvate exporter is currently not known; it is feasible that pyruvate crosses the mitochondrial membrane as pyruvic acid by diffusion because at least at high concentrations, pyruvate can move by diffusion in vesicles [38]. UCP1, upregulated in both NAD-ME and NADP-ME plants maintains redox poise in photosynthetic tissues [42], but no other known mitochondrial transport protein is consistently upregulated in the C_4 species assayed by RNA-seq.

The third decarboxylation module is run by PEP-CK and occurs in the cytosol (Figure 1f). The action of PEP-CK requires one ATP per reaction which is likely supplied by the mitochondria as the plastidic ATP exchanger is not relevant during day-time metabolism [43]. Export of ATP is mediated by AAC [44], a gene transcriptionally abundant in many species (Table 1) but specifically upregulated in the C_4 species *Megathyrsus maximus* which relies to a large degree on PEP-CK [8]. Alternatively, PEP may be metabolized to pyruvate directly by cytosolic pyruvate kinase which will supply the ATP for the decarboxylation reaction making the ATP export from mitochondria unnecessary.

Transfer acid generation module

The OAA generated by PEPC in the mesophyll is immediate converted to malate or aspartate before transfer and the pyruvate generated by MEs in the bundle sheath (Figure 1d and e) can be converted to alanine before transfer. The localization and role of the transfer acid generation is not well characterized.

Most C_4 species analyzed harbor a transcriptionally highly abundant alanine aminotransferase (AlaAT) (Table 1) [5••,7,8] of which the localization is unclear. In dicots, the AlaAT appears to be organelle-localized in which case additional transport processes were required. In maize, AlaAT is evenly distributed between mesophyll and bundle sheath (Table 1).

Plants contain AspAT and MDH in chloroplasts, mitochondria and the cytosol and each can be upregulated and/or abundant in C_4 species (Table 1). All C_4 plants analyzed to date which use NADP-ME based decarboxylation show chloroplast-dependent conversion reactions (Figure 1g). OAA is reduced in the chloroplasts after counter-exchange with malate by dicarboxylate translocator 1 (DiT1) across the plastid envelope [45]. Alternatively, the OAA is converted to aspartate (Asp) in chloroplasts by plastidic aspartate transaminase (AspAT) [12,46] after import by DiT2 [36] in exchange for aspartate. Both enzymes and transporters are moderately upregulated [7]; in maize, AspAT, MDH, and DiT1 are mesophyll specific while DiT2 is bundle sheath specific (Table 1). The transamination reaction requires an amino group donor in the chloroplast which may come directly from the N assimilation or from a plastidic AlaAT reaction realizing the regeneration of pyruvate. In the mixed PEP-CK/NAD-ME *M. maximus*, the cytosolic AspAT and MDH are abundant (Table 1) [8]. The NAD-ME species *G. gynandra* highly expresses a mitochondria localized AspAT [5••,13]. It is unclear which transport protein catalyzes the exchange of aspartate and OAA across the mitochondrial envelope (Figure 1h); the DIC does not transport amino acids [41], and for DTC the transport of amino acids was not tested [47].

Metabolic integration

The C_4 cycle runs atop C_3 metabolism, interacts with it, and creates different environments in mesophyll and bundle sheath cells all of which require fine tuning. For example, abundant expression of TPT [5••,16] (Table 1) couples the parts of the Calvin Benson Bassham cycle localized in the mesophyll and bundle sheath [24•,48]. The shuttle may also transfer ATP and reducing equivalents in the form of triosephosphates [16]. C_4 metabolism creates large pools of the transfer metabolites which need to be protected from the underlying metabolism, for example by reducing entry in the TCA cycle via pyruvate dehydrogenase complex kinase [8]. Integration into the underlying metabolism may be different not only based the use of decarboxylation enzyme in each species but may also carry further species specific solutions for metabolic adjustment. Different comparative RNA-seq papers have reported a range of possible adaptations including reduction of photorespiration in all systems studied to date [8,9•], reduction in amino acid and protein synthesis in some species [5••,7,9•] and changes in amino acid metabolism for *G. gynandropsis* and *M. maximus* [8]. The reduction in photorespiratory gene expression was suggested as the molecular change which fixes the C_4 trait [49]. The connection of the two cell types and efficient intercellular transfer is critical [8], but so far understudied.

Summary and outlook

RNA-seq has contributed to the molecular identification of novel [28••] and known [5••,8] transporters of the C_4 cycle and identified the enzymes of the C_4 cycle at the molecular level in many species (Table 1). The blueprint of the C_4 cycle is complete for NAD-ME and PEP-CK module (Figure 1e and f) [8], but less well defined for Andropogonae NADP-ME species as the mesophyll pyruvate transporter remains unknown (Figure 1b). Although the genes involved in transfer acid generation appear defined, their intra-cellular and inter-cellular localization and potential need for additional transporters

remain unclear. Additional RNA-seq experiments may define the modules ever more clearly for new C_4 species while combined RNA-seq and genome-seq experiments will move the analyses to the next step: identifying the regulatory circuits underlying the high abundance detected by RNA-seq. Expression patterns of C_4 cycle genes are similar to those of photosynthetic genes [30,50,51] already suggesting a common regulatory system.

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