Unknown components of the plastidial permeome

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INTRODUCTION

Both, plastids and mitochondria are of endosymbiotic origin and play a pivotal role in energy supply and metabolism of plant cells. Both organelles harbor a plethora of metabolic processes and play a pivotal role in energy supply and metabolism of plant cells.

Beyond their role in photosynthesis plastids provide a plethora of additional metabolic functions to plant cells. For example, they harbor complete biosynthetic pathways for the de novo synthesis of carotenoids, fatty acids, and amino acids. Furthermore plastids contribute important reactions to multi-compartmentalized pathways, such as photosynthesis or plant hormone syntheses, and they depend on the import of essential molecules that they cannot synthesize themselves, such as ascorbic acid.

Recently it was shown that exchange of non-polar substrates between the plastid and the ER can proceed without the involvement of specific transport proteins by a membrane hemi-fusion mechanism (Mehrshahi et al., 2013). However, more than 100 putative plastidial transporters have been identified by in silico methods (Weber et al., 2005; Armbruster et al., 2011) and various chloroplast inner and outer envelope proteins that were detected by proteomics or transcriptomics are still awaiting functional characterization (Braithagam et al., 2008; Braithagam and Weber, 2009; Manandhar-Shrestha et al., 2013).

Hence, assuming that the abovementioned estimates are sensible, approximately 75% of the plastidial translocators remain unidentified as molecular entities. An example for the obstacles on the way to success is the recently identified photorespiratory glycolate glycerate transporter (Pick et al., 2013). The existence of this transport protein and its transport mode were established by biochemical assays already in the 1980s (Howitz and McCarty, 1985a,b, 1986). Nevertheless it took several decades to eventually link the biochemical function to a molecular entity, which became possible through multivariate statistical analyses of Arabidopsis gene expression datasets (Pick et al., 2013).

In this review we will focus on a subset of these unknown transporters, hence summarizing a selection of biochemical pathways with predicted metabolite transport steps over the plastid envelope (Figure 1).

TRANSPORT OF ASCORBIC ACID

In plants, ascorbate is present in all tissues and cell compartments (Zeichmann et al., 2011). Up to four routes lead to the formation of ascorbate, including the l-galactose-pathway, the animal-like pathway via myo-inositol/glucuronate, the salvage pathway via l-galactonate, and the l-gulose-pathway. Among these four routes the biosynthetic route via l-galactose, also called the Smirnoff–Wheeler pathway, is the major pathway in many

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FIGURE 1 | Schematic representation of biochemical pathways with predicted metabolite transport steps over the plastid envelope discussed in this review. Ascorbate is exclusively produced in mitochondria and transported into plastids. IPP is either (A) exported in a Ca^{2+}-gated IPP/proton symport mechanism or (B) transport absolutely depends on binding of phosphorylated small molecular weight compounds (psmwc) to a regulatory side on the trans side of the membrane. Nitrite transport likely involves binding of plastidic PII protein. Sulfate is imported by a sulfate/proton cotransporter and is partially mediated by SULTR3:1, presumably involving other members of the SULT3 family or a second independent transport system. CA is produced from CP in the plastid and exported to the cytosol. Further enzymatic steps in the cytosol and mitochondria lead to the formation of UMP. Fatty acids are synthesized in the plastid, attached to an ACP and exported either (C) involving a transport protein and binding of ACS in the outer envelope or (D) by facilitated diffusion leading to formation of FA–CoA. The green line represents the plastid outer envelope membrane (OE). The black line represents the plastid inner envelope membrane (IE). Black arrows indicate enzymatic steps. Gray squares represent transport proteins. OE, plastid outer envelope membrane; IE, plastid inner envelope membrane; psmwc, phosphorylated small molecular weight compounds; IPP, Isopentenyl diphosphate; PII, plastidic PII protein; CP, Carbamoyl phosphate; CA, Carbamoyl aspartate; UMP, uridine monophosphate; FA, free fatty acids; FA–ACP, fatty acid – Acyl carrier protein; ACS, acyl-coenzyme A (CoA) synthetase.

plants (Wheeler et al., 1998; Gatzek et al., 2002; Laing et al., 2007; Bulley et al., 2009). In this pathway D-glucose is converted via the intermediates GDP-D-mannose and L-galactose to L-ascorbate. Only recently the last missing enzyme in this pathway was identified, namely VTC2, a GDP-L-galactose phosphorylase (Laing et al., 2007). VTC2 was shown to be the rate-limiting step in ascorbate biosynthesis and overexpression of the VTC2 orthologous from kiwifruit (Actinidia spp.) in Arabidopsis resulted in an up to fourfold increase in ascorbate levels (Bulley et al., 2009). In addition, transient overexpression of VTC2 in Arabidopsis revealed its function as a major control point in light/dark regulation of ascorbate biosynthesis (Yoshimura et al., 2014). Except the last enzymatic step of ascorbate biosynthesis, the conversion of L-galactono-1,4-lactone to L-ascorbate by L-galactono-1,4-lactone dehydrogenase (GLDH) on the inner mitochondrial membrane all enzymatic reactions take place in the cytosol. Among the other pathways the salvage pathway via L-galactono-1,4-lactone is the best-characterized ascorbate transporter resides in the plasma membrane. It was characterized in isolated plasma membrane vesicles of Phaseolus vulgaris L., indicating an ascorbate/dehydroascorbate antiport mechanism (Horemans et al., 1996). Transport over the plastid envelope was determined biochemically. Uptake assays using intact isolated chloroplasts from Spinacia oleracea and Pisum sativum revealed that ascorbate is taken up by a saturable carrier that has a very low affinity ($K_m = 20$ mM), and does not transport glucose (Beck et al., 1983; Foyer and Lelandais, 1996).
Also, a reduction of the import of the MEP pathway pretriose phosphates and pyruvate in plastids via the 1-deoxy-D-xylulose 5-phosphate/methylerythritol phosphate (DOXP/MEP) pathway was shown, suggesting transporter mediated exchange of both pathways was shown, indicating a transporter mediated and regulated uptake of nitrite (Brunswick and Cresswell, 1988a,b). A nitrite transporter candidate, NitrIL, was shown to be chloroplast localized and Arabidopsis knockout mutants exhibited a five times higher accumulation of nitrite in leaves compared to wild type plants, pointing to a nitrite transport function of NitrIL (Sugiura et al., 2007). Moreover, it was shown that the plastidic PII protein is involved in nitrite uptake through a yet unknown mechanism (Ferrario-Mery et al., 2008). However, interaction of PII with NitrIL and biochemically direct nitrite uptake by NitrIL still remains to be demonstrated. In addition, a recent study localized NitrIL to the plasma membrane in grapevine and Arabidopsis, which makes it unlikely that this protein represents the plastidial nitrite transporter (Pike et al., 2014).

Recently AtNITR2;1, a transporter belonging to the HPP protein family, was identified as a new nitrite transporter candidate (Maeda et al., 2014). AtNITR2;1 is localized to the plastid IE (Ferro et al., 2003), expressed in shoot and root, and seems to be conserved in vascular plants (Maeda et al., 2014). When expressed in NIT2;1, the nitrite transport-less mutant of the cyanobacterium Synechococcus elongatus, AtNITR2;1 exhibited a high affinity for nitrite $K_m = 13 \mu M$. Additionally, nitr2;1 Arabidopsis knockout mutants showed reduced uptake of nitrite into isolated chloroplasts compared to WT levels. From these experiments it was calculated that NITR2;1 accounts for $\sim 60\%$ of nitrite uptake at $50 \mu M$ external nitrite. However, since nitr2;1 mutant plants did not exhibit a visible phenotype when grown on nitrate as sole nitrogen source (Maeda et al., 2014), the physiological role of NITR2;1 still needs to be further investigated.

Sulfate is reduced and assimilated into cysteine and methionine in the chloroplast. Hence, sulfate has to be transported from the cytosol into chloroplasts. Transport studies revealed a sulfate import via a proton/sulfate co-transporter (Buchner et al., 2004). Recently a functional plastidic sulfate transporter SULTR3;1 was identified by knockout mutant analysis in Arabidopsis (Cao et al., 2013). SULTR3;1 belongs to the SULTR3 subfamily of plant sulfate transporters that is composed of five members (Takahashi et al., 2000). In yeast complementation studies SULTR3;1, SULTR3;2, and SULTR3;3 failed to complement the phenotype of the yeast mutant lacking both sulfur transporters (Takahashi et al., 2000), probably due to incorrect targeting of the proteins. However, in organello transport assays confirmed the proton/sulfate symport but revealed the existence of further plastidic sulfate transporters in addition to SULTR3;1 (Cao et al., 2013). In silico analysis revealed similarities in genomic organization of SULTR3;1.
and Sultr3;2 pointing to functional redundancy of SULTR3 subfamily members (Takahashi et al., 2000; Cao et al., 2013). Absence of strong phenotypes in single mutant plants additionally point to functional redundancy (Zuber et al., 2010). However, array data point to a low expression of SULTR3;2 throughout the plant (EPF browser Arabidopsis, Winter et al., 2007) implying the possibility of an additional transport system independent of SULTR3 family members. Further analysis of sultr3;1 sultr3;2 double and sultr3;1 sultr3;2 sultr3;3 sultr3;4 quadruple mutants may reveal how these transporters contribute to plastidic sulfate import.

**PYRIMIDINES**

Nucleotides, including purines and pyrimidines, are crucial components for plant primary and secondary metabolism and development. Pyrimidine de novo synthesis is a highly compartmentalized pathway involving enzymatic reactions in plastids, mitochondria, and the cytosol. The initial steps take place in plastids, eventually leading to the formation of carbamyl aspartate (CA; Shibata et al., 1986; Chen and Slocum, 2008). In contrast to previous assumptions, where the conversion from CA to dihydroorotate (DHO) by DHOase took place in the plastids, protein-GFP fusion analysis could clearly localize the DHOase to the cytosol (Witz et al., 2012). This implies that CA has to be exported from plastids into the cytosol. In addition to de novo synthesis pyrimidines can also be synthesized via the less energy consuming salvage pathway. Presumably de novo synthesis in plants occurs in dividing cells and growing tissues where a high amount of nucleotides is required, whereas the salvage pathway operates in non-growing cells and mature tissues. Two studies revealed that the salvage pathway plays an essential role in the supply of pyrimidines to the plant (Mainguet et al., 2009; Chen and Thelen, 2011). Here it was shown that single mutant plants lacking plastidic uracil phosphoribosyltransferase (UPRT; Mainguet et al., 2009) and double mutant plants lacking both plastidic isoforms of uridine kinase (UKL1 and UKL2; Chen and Thelen, 2011) display dwarfish and chlorotic phenotypes. Recently the plastidic uracil transporter PLUTO (plastidic nucleobase transporter) that is involved in the salvage pathway was identified (Witz et al., 2012). However, the transport mode of CA export during de novo synthesis and a transport protein remain unknown. As CA is a modified amino acid, transport through the DI T protein family, members of the plastidic amino acid permease (AAP) family, or the plast proteinpreprotein and amino acid transporter (PRAT) superfamily might be possible (Renne et al., 2003; Weber et al., 2005; Murcha et al., 2007).

**AMINO ACIDS**

Amino acids are the building blocks of enzymes and proteins and fulfill various additional functions in plants. They act as nitrogen donors for a variety of essential compounds and play an indispensable role in important processes like photorespiration. Plants are able to de novo synthesize all 20 proteogenic amino acids, while several enzymes involved in the biosynthesis of amino acids reside in the plastids and many amino acids are produced here (Kirk and Leech, 1972; Ravanel et al., 2004). Recently it was shown that the enzymatic steps of amino acid biosynthesis in plastids predominantly consist of non-cyanobacterial enzymes (Reyes-Prieto and Moustafa, 2012). Since protein synthesis occurs in the cytosol, plastids, and mitochondria, a high flux rate of amino acids across the plastid envelope is required, at least during developmental stages that are associated with high rates of protein biosynthesis. Hence, specific import and export proteins are required. To date, the only characterized plastidic IE amino acid transporter is AtDiT2.1 (Renne et al., 2003). AtDiT2.1 mediates the exchange of glutamate/malate and also accepts the amino acid aspartate as transport substrate (Werner-Washburne and Keegstra, 1983, 1985). Based on several studies, in particular proteomics, transporter candidates for plastidic amino acid transporters were identified (Kleffmann et al., 2004; Brautigam et al., 2008). For example members of the PRAT superfamily PRAT1 and PRAT2 were shown to be plastid-localized (Murcha et al., 2007) but their transport function remains to be demonstrated.

**FATTY ACIDS**

In plants, plastids are the site of de novo fatty acid synthesis. Following synthesis, fatty acids are activated by a plastid localized acyl carrier protein (acyl-ACP) and are thus prepared for further complex lipid assembly (Ohlrogge et al., 1979). In Arabidopsis leaf mesophyll cells 62% of the chloroplastic produced fatty acids are exported (Browse et al., 1986). In non-photosynthetic tissues and developing seeds of all plants even 90% of the fatty acids are exported (Browse et al., 1993). However, the molecular mechanism of fatty acid export from plastids is still controversial. It was proposed that membrane contact sites between plastids and endoplasmatic reticulum were involved (Andersson et al., 2007a,b) although experimental evidence is still missing. It has been shown, that before leaving the plastid, attaching the fatty acid to an acyl-ACP and fatty acid activation by acyl-coenzyme A (CoA) synthetase (ACS) forming fatty acyl-CoAs is mandatory (Ohlrogge et al., 2000). Both, acyl-ACP thioesterase and ACS were shown to be plastid localized with a clear localization of ACS to the plastid outer envelope (Anderssen and Keegstra, 1983; Schnurr et al., 2002; Breuers et al., 2012). Recent rapid kinetic label experiments suggested that phosphatidylcholine participates in the export of newly synthesized acyl chains from plastids (Tjellstroem et al., 2012). But it is still under debate if fatty acid export is mediated by facilitated diffusion or specific transport proteins. The latter mechanism was already shown to occur in bacteria and yeast and involve activity of ACS proteins (Black and DiRusso, 2003). Recently it was also shown that the cyanobacterial ACS SLR1609 mediates the transport of fatty acids across a biological membrane (von Berlepsch et al., 2012). Transporter candidates in Arabidopsis were previously suggested but biochemical evidence for their involvement in fatty acid transport remains elusive (Koo and Ohlrogge, 2002).

**CONCLUSION**

Over the past decade substantial effort was invested in the identification of additional plastid envelope metabolite translocators as molecular entities. These recent studies led to the molecular identification of several transporters that were previously predicted...
on the basis of biochemical evidence, such as the plastidal pyruvate and glycolate/glycerate transporters (Furumoto et al., 2011; Pick et al., 2013). Recent “omics” and bioinformatics approaches, such as coexpression analysis, lead to the emergence of further candidates for putative transporters in the last years that, however, still await functional characterization (Brautigam et al., 2008; Brautigam and Weber, 2009; Majeran et al., 2010; Bordyeh et al., 2013; Manandhar-Shrestha et al., 2013).

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