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Proteomic Analysis of the Proplastid Envelope Membrane Provides Novel Insights into Small Molecule and Protein Transport across Proplastid Membranes

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ABSTRACT Proplastids are undifferentiated plastids of meristematic tissues that synthesize amino acids for protein synthesis, fatty acids for membrane lipid production, and purines and pyrimidines for DNA and RNA synthesis. Unlike chloroplasts, proplastids depend on supply, with reducing power, energy, and precursor metabolites from the remainder of the cell. Comparing proplastid and chloroplast envelope proteomes and the corresponding transcriptomes of leaves and shoot apex revealed a clearly distinct composition of the proplastid envelope. It is geared towards import of metabolic precursors and export of product metabolites for the rapidly dividing cell. The analysis also suggested a new role for the triosephosphate translocator in meristematic tissues, identified the route of organic nitrogen import into proplastids, and detected an adenine nucleotide exporter. The protein import complex contains the import receptors Toc120 and Toc132 and lacks the redox sensing complex subunits of Tic32, Tic55, and Tic62, which mirrors the expression patterns of the corresponding genes in leaves and the shoot apex. We further show that the protein composition of the internal membrane system is similar to etioplasts, as it is dominated by the ATP synthase complex and thus remarkably differs from that of chloroplast thylakoids.

Key words: Membrane biochemistry; proteomics; transporters; chloroplast biology; membrane proteins; transcriptome analysis.

INTRODUCTION

The apical and lateral meristems of plants serve as reservoirs for undifferentiated stem cells that permit indeterminate plant growth. In all meristems, only very few cells are actual stem cells, while the remainder of the meristem consists of cells that will continue to divide for a finite number of divisions. Meristematic cells are usually surrounded by a thin primary cell wall and they lack a central vacuole and other differentiated features. Since meristems do not contain photosynthetically active chloroplasts, but undifferentiated proplastids, they are sink tissues, which rely on the source tissues for supply with reduced carbon. Although undifferentiated, proplastids support cell growth and division by providing building blocks for the cells in the form of branched chain and aromatic amino acids, fatty acids, and lipids, as well as nucleotide precursors. Proplastids can differentiate into all plastid subtypes, such as chloroplasts, chromoplasts, and leucoplasts (Kirk and Tilney-Bassett, 1978). Since meristematic tissues are usually small, they have not been amenable to analysis by methods requiring large amounts of tissue such as proteomics. In contrast, cauliflower (*Brassica oleracea* ssp. *botrytis*) is an excellent model for proteomics of meristematic tissues. The head of cauliflower, named the 'curd', represents a highly branched abnormally proliferated meristem before floral transition (Kieffer et al., 1998). The branch primordia only grow for a short time until they themselves become apical meristems and produce additional branch primordia (Kieffer et al., 1998). This phenotype has been genetically at least partially tied to floral identity

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gene mutations (Smith and King, 2000), although most of the phenotypic variability of *B. oleracea* remains unexplained (Labate et al., 2006). Only much later in development do these abnormally proliferating meristems initiate floral development (Kieffer et al., 1998). Before initiation of floral development, the curd remains an excellent source of meristematic or near meristematic tissues and organelles. The plastids of cauliflower curd tissue (Journet and Douce, 1985) resemble proplastids of other meristematic tissues (summarized in Kirk and Tilney-Bassett, 1978). They are able to undergo differentiation into chloroplasts as demonstrated by 'greening' of the curds if the cauliflower is not properly covered either by its own leaves or foil. Cauliflower curd proplastids can also differentiate into chromoplasts (Crisp et al., 1975; Lu et al., 2006). Like the differentiated plastid types, proplastids are surrounded by two envelope membranes, called the outer and the inner envelope membranes. Within these boundaries, a protein-rich matrix, the stroma, and a rudimentary internal membrane system are contained (Journet and Douce, 1985). The internal membrane system differentiates into a prolamellar body in etioplasts or into a thylakoid system in chloroplasts (Kirk and Tilney-Bassett, 1978).

Although the phloem stream is capable of supplying a number of metabolites to the meristem (Corbesier et al., 2002; Weibull and Melin, 1990; Zhu et al., 2005), the only model for proplastids, plastids of cell cultured tobacco cells (BY-2 cells) are enriched in enzymes for amino acid biosynthesis, indicating a metabolically active plastid (Baginsky et al., 2004). Biochemical analyses of cauliflower curd proplastids have revealed that they import glucose-6-phosphate and that they store carbon as starch (Emes and Neuhaus, 1997; Journet and Douce, 1985; Neuhaus and Emes, 2000). A large supply of carbon is needed not only to prepare the proplastid for differentiation, but also for anabolic reactions such as fatty acid, purine and pyrimidine, or amino acid biosyntheses. Organic nitrogen is also delivered to the meristem by the phloem, but it is unknown how proplastids import it (Emes and Neuhaus, 1997; Neuhaus and Emes, 2000). Since the proplastid is the sole cellular source for a number of essential metabolites for the dividing and growing meristematic cells, efficient exchange of metabolites across the envelope necessitates the presence of metabolite transport proteins.

In this work, we focused on the envelope proteome of cauliflower curd proplastids to understand its adaptations to proplastid-specific metabolism. The transport protein complement indicated that proplastids are active cellular factories, which import precursor metabolites and export products such as amino acids, fatty acids, and nucleotides. The proplastids did contain not only a distinct set of transport proteins, but also a subset of protein import complex components as well as a distinct subset of thylakoid resident proteins very unlike the thylakoid contamination found in chloroplast envelope proteomics. The steady state mRNA levels corresponding to the proteins identified in this work are generally higher in the shoot apex in comparison to mRNAs encoding proteins that were identified in leaf chloroplast envelope proteomics. In summary, the proplastid envelope is specifically adapted to both producing building blocks for the cell as well as differentiating upon external cues.

RESULTS

Overall Membrane Proteome Analysis

Proteomic analysis of cauliflower curd plastid envelope membranes identified 226 distinct proteins (Table 1). This number was slightly lower compared to 289 proteins identified in a previous study of pea chloroplast envelope membranes performed with identical protocols on the same machine (Bräutigam et al., 2008b), which was re-analyzed the same way as the cauliflower curd plastid envelopes (Supplemental Tables 1 and 2). The proplastid envelope sample allowed the identification of 15 known and 13 putative transport proteins, 6.6% and 5.8% of the total proteins identified, respectively, whereas the chloroplast envelope proteome sample allowed only the detection of 4.2 and 4.8%, respectively. Overall, approximately one-third of the proteins contained at least one predicted transmembrane helix and this figure was similar between both proteome samples. When mapping the expression ratios of the orthologous *Arabidopsis* proteins to all the proteins identified in the chloroplast and proplastid membrane sample, the median of the proplastid sample was shifted towards shoot apex expression (Figure 1). Chloroplast membrane proteins showed greater scattering than those of the proplastid membrane sample.

Transport Proteins

The membrane transport proteins of cauliflower curd envelopes differed from those identified in other plastid proteome projects (Table 2). On the plastid envelope, only one ABC transport system has been well characterized. Two components of this TGD ABC transporter complex, which moves phosphatidic acid (Awai et al., 2006; Lu et al., 2007; Xu et al., 2005), were identified in the proplastid envelope. This transport complex or at least parts of this complex have already been identified in chloroplast proteome projects and in etioplast proteomics, but not in proplastid proteomics (Table 2). One other partial ABC

Table 1. Membrane Protein Identification in Proplastid and Chloroplast Envelopes from *B. oleracea* and *P. sativum*.

	Total	Membrane proteins	Known transport proteins	Putative transport proteins
Proplastid envelope	226	81	15	13
Chloroplast envelope	289	98	12	14

The chloroplasts envelope was reanalyzed from Bräutigam et al. (2008b).

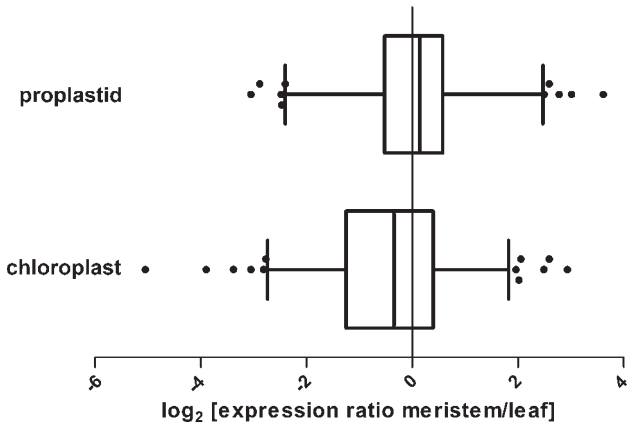

Figure 1. Box-Whisker Plot of the Expression Patterns of the Homologous *Arabidopsis* Proteins Identified in Proplastid and Chloroplast Envelopes.

The box shows the median and the adjacent quartiles, the whiskers extend to 2.5 and 97.5% of the data, respectively. Positive $\log_2$ values indicate higher expression in the meristem compared to leaves.

transporter protein, NAP8, was also identified in proplastid and chloroplast proteomics.

Three of the four characterized members of the phosphate translocator family were identified—the triosephosphate phosphate translocator (TPT) (Loddenkötter et al., 1993), the phospho*enol*pyruvate phosphate translocator (PPT) (Fischer et al., 1997), and two isoforms of the glucose-6-phosphate phosphate translocator (GPT) (Kammerer et al., 1998)—while the pentosephosphate translocator (XPT) (Eicks et al., 2002) was not detectable. Although the GPT isoforms are very similar, diagnostic peptides of both isoforms could be identified. In contrast, GPT is absent from chloroplast proteomics experiments (Table 2), whereas XPT could be identified in at least one chloroplast envelope proteome project (Supplemental Table 2).

Two ATP/ADP exchangers, NTT1 and NTT2 (Neuhaus et al., 1997), were identified with diagnostic peptides each. One phosphate transporter, a member of the major facilitator superfamily, which may exchange phosphate against protons or sodium (Guo et al., 2008) was found. Six members of the mitochondrial carrier family (MCF) were identified. The mitochondrial di- and tricarboxylate carriers (Picault et al., 2002) are likely mitochondrial contaminations (Supplemental Table 1). The Brittle1-like protein (Kirchberger et al., 2008) transports adenine nucleotides as a uniporter. The phosphate transporter PHT3-1 transports phosphate either in symport with OH- or in antiport mode with protons (Takabatake et al., 1999). One of the two S-adenosyl methionine transporters (SAMCs) (Bouvier et al., 2006) was identified as well as two mitochondrial carrier family members, one of which branches with the SAMCs and the basic amino acid transporting MCF members and one of which branches with the adenine nucleotide transporters (Picault et al., 2004) (Table 2). The sample also contained both plastidic dicarboxylate translocators, DiT1, which mainly transports 2-oxoglutarate and malate (Weber et al., 1995), and DiT2, which mainly transports glutamate and malate (Renne et al., 2003; Taniguchi et al., 2002).

A number of proteins of unknown functions were identified that could be categorized in different transport protein families: three putative bile acid sodium symporter (BASS) family proteins, two sugar transporter family proteins, two proteins of the Tim17/Tim22/Tim23 family (Murcha et al., 2007), and one member of the MATE efflux protein family (www.tcdb.org/index.php) (Table 2). Two putative ion transporters, the putative potassium proton transporter, KEA1 (Maser et al., 2001), and a putative magnesium transporter, MGT10 (Li et al., 2001), were also present in the proplastid envelope sample.

Of the known outer envelope transport proteins, OEP 16, an amino acid transporter (Pohlmeyer et al., 1997), OEP 24, a transport protein with broad selectivity for metabolites such as sugarphosphates, charged amino acids, ATP and dicarboxylates (Pohlmeyer et al., 1998), and OEP 37, a cation selective channel (Goetze et al., 2006), were found in the proplastid proteome sample.

Analysis of the virtual plastid envelope transcriptome revealed that only transcripts encoding seven known and putative envelope transport proteins were increased more than twofold in shoot apex samples compared to leaf samples extracted from publicly available *Arabidopsis* expression arrays: three ABC carriers of unknown function, At5g52860, At1g15210, and At2g28070, one member of the bile acid sodium symporter (BASS) family, At4g12030, one member of the mitochondrial carrier family, At5g64970, a member of the MATE efflux family, At4g38380, and the MCF member Brittle1-like protein (Figure 2). The corresponding proteins were detected for three of these by proplastid envelope proteomics: the MCF member, the MATE efflux family protein, and Brittle1-like (Table 2).

The Protein Import Complex

The protein import complex components identified in proplastid envelopes included all three currently known import receptors, Toc120, Toc132, and Toc159, two smaller GTPases, Toc33 and Toc34, and the main protein import channel through the outer envelope, Toc75-III, as well as Toc75-V (Supplemental Table 1) (Jarvis and Robinson, 2004; Schnell, 2000; Soll and Schleiff, 2004). Of the inner envelope complex, Tic110, Tic20, Tic40, and Tic22, as well as the import chaperone Hsp93 (also called ClpC), were found (Supplemental Table 1) (Jarvis and Robinson, 2004; Schnell, 2000; Soll and Schleiff, 2004). In chloroplast envelope proteomics of leaf samples, Toc120 and Toc132 have not been identified to date (Bräutigam et al., 2008a, 2008b; Ferro et al., 2003; Froehlich et al., 2003; Kleffmann et al., 2004, 2007). For the inner envelope complex, Tic32, Tic55, and Tic62 could only be identified in chloroplast proteomics (Supplemental Table 2) (Bräutigam et al., 2008b). The expression pattern of the protein import complex components was analyzed in detail and compared to the identification of components in chloroplast and proplastid proteomics (Figure 2). The proteins with the highest shoot apex/leaf expression ratio, the alternative import receptors Toc120 and

Table 2. Known and Putative Transport Proteins of the Proplastid Envelope Proteome.

AGI (* AGI of *Arabidopsis* homolog)	Gene identification	Substrates (* putative substrates)	Involved in ... (* putatively involved in ...)	Log_2 (ratio of shoot apex to leaf expression)	Also identified in other plastid proteome projects
* AT3g20320	TGD2	Phosphatidic acid	Lipid remodeling	0.10	a, b, c, e, f, g,
AT1G65410	TGD3	Phosphatidic acid		−0.21	a, e,
AT4G25450	Non-intrinsic ABC type protein (NAP8)	?		−0.75	a, b, c, e, f,
AT5G46110	TPT	Triosephosphate, 3-PGA, phosphate	Export of triosephosphates, redox shuttling	−1.38	a, b, c, d, e, f, g,
AT5G33320	PPT1/ CUE1	PEP	PEP import for shikimate pathway	0.84	a, b, c, d, e, f, g,
AT5G54800	GPT1	Hexosephosphate, phosphate	Redox import, carbon skeleton import	0.40	h
AT1G61800	GPT2	Hexosephosphate, phosphate	Redox import, carbon skeleton import	−1.72	h
AT1G80300	NTT1	ATP, ADP	Energy shuttle	1.09	b, c, e, f, g, h
AT1G15500	NTT2	ATP, ADP	Energy shuttle	1.09	a, b, c, g, h
AT4G32400	Brittle1-like	ATP	Export of adenylates	1.43	a, f,
AT4G39460	SAMC1	S-adenosylmethionine, S-homocysteine	Providing methylgroups for biosynthetic pathways	0.22	a, c, e
AT5G14040	PHT3-1	Phosphate, * protons	* Coupling of phosphate to the proton gradient	0.76	d, e, h
* AT4G00370	ANTR2/ PHT4-4	Phosphate, * sodium	* Coupling of sodium via phosphate to the proton gradient	−0.12	a, e, f,
AT5G64970	Mitochondrial carrier family protein	?		1.10	e
AT5G42130	Mitochondrial carrier family protein	?		−0.29	a, e, f
AT5G12860	DiT1	2-OG, malate	Shuttling organic nitrogen	−1.31	a, b, c, d, e, f, h
AT5G64290	DiT2.1	Glutamate, malate		−1.15	a, b, c, d, e, f
AT2G26900	Bile acid:sodium symporter family protein	* Anion, * sodium		0.91	c, e
AT4G22840	Bile acid:sodium symporter family	* Anion, * sodium		−0.17	
* AT3G56160	Bile acid:sodium symporter family protein	* Anion, * sodium		−0.47	a, b, e, f
AT5G24650	Tim17/Tim22/Tim23 family protein	?		0.55	a, b, c, e, f
AT4G26670	Tim17/Tim22/Tim23 family protein	?		0.22	a, c, f
AT5G59250	Sugar transporter family protein	?		−1.37	a, b, c, d, e, f, g
AT5G16150	Sugar transporter family protein	?		0.18	b, c, f, g
* AT4G38380	DTX45; MATE efflux family protein	* Complex metabolites		1.61	a
AT1G01790	KEA1	* Potassium, * protons	* Ion balance	−1.65	a, b, c, d, e, f, g, h
AT5G22830	MGT10	* Magnesium	* Magnesium import	−0.50	a, b, e
At2g28900	OEP 16	Amino acids	Amino acid export	−1.87	a, b, c, d, e, f
At5g42960	OEP 24	Charged solutes	Multiple pathways	0.37	a, b, d, e, f
At2g43950	OEP 37	?, cation selective		0.35	b, c, e, f

The expression pattern was determined as described in Methods; positive values indicate expression higher in the shoot apex for the corresponding *Arabidopsis* proteins. Orthologs of the proteins were also identified in other proteome projects: a, Zybailov et al. (2008); b, Froehlich et al. (2003); c, Ferro et al. (2003); d, Kleffmann et al. (2004); e, Bräutigam at al. (2008a); f, Bräutigam et al. (2008b); g, von Zychlinsky et al. (2005); h, Baginsky et al. (2004).

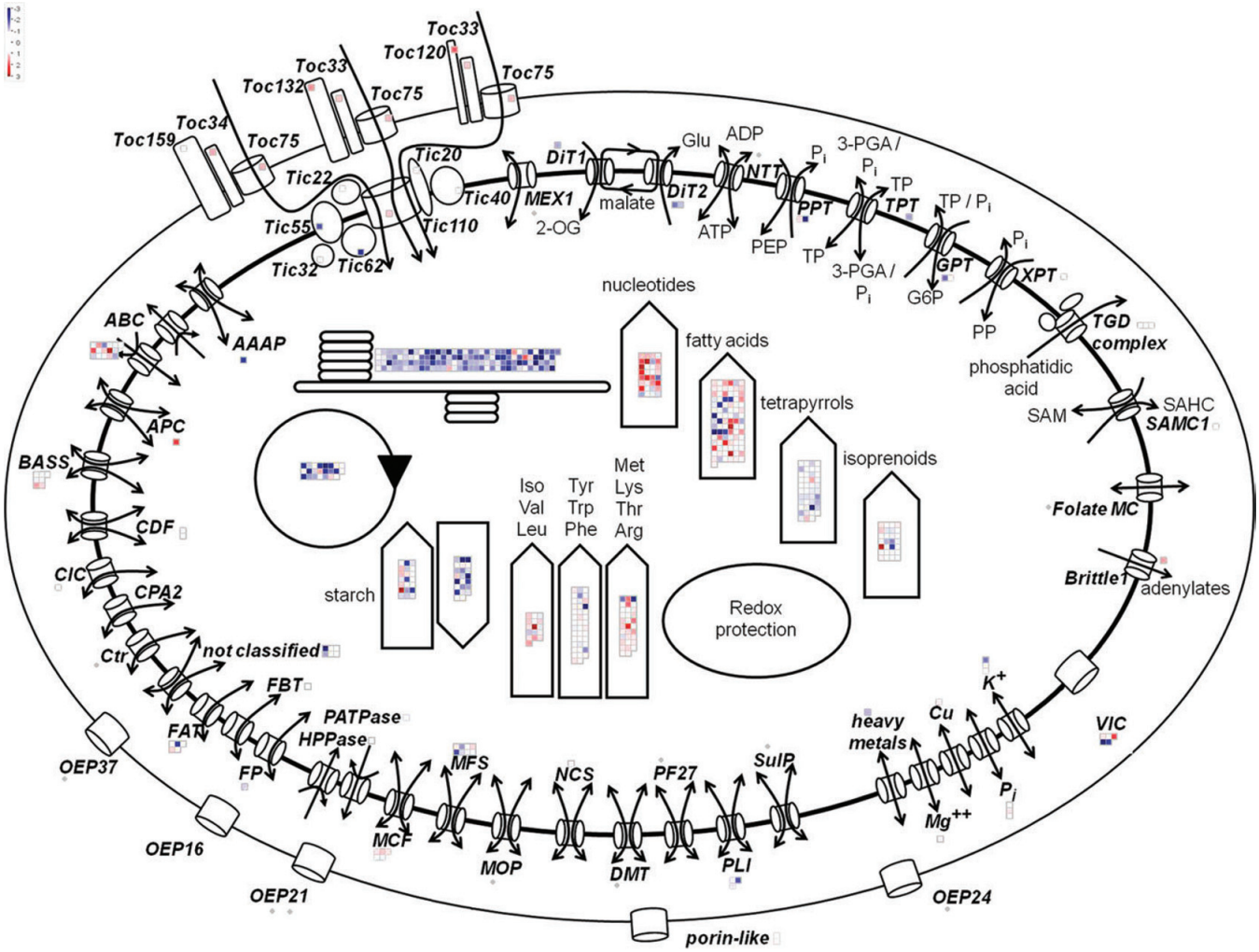

Figure 2. Mapman Representation of Expression Pattern for Putative and Known Transport Proteins and Members of the Protein Import Complex.

Higher expression in the shoot apex is visualized as increasingly dark red color, whereas higher expression in the shoot is indicated by darker blue color. To visualize the meristematic metabolism, selected pathways are plotted within the virtual plastid, with arrow pointing upward indicating synthesis and downward indicating breakdown, starch biosynthesis (mapman bin 2.1.2), and breakdown (mapman bin 2.1.1), nucleotide biosynthesis (mapman bin 23.1), fatty acid biosynthesis (mapman bin 11.1), tetrapyrrol biosynthesis (mapman bin 19), isoprenoid biosynthesis (mapman bin 16.1.1), and aminoacid biosynthesis (mapman bins 13.1.3, 13.1.4, and 13.1.6). The circle represents the reductive pentosephosphate cycle (mapman bin 1.3) and the thylakoid drawing represents genes of the light reaction (mapman bin 1.1). All files needed to plot the virtual plastid in Mapman are available as supplemental materials. AAAP, amino acid auxin permease family; ABC, ATP-binding cassette (ABC) superfamily; APC, amino acid-polyamine-organocation family; BASS, bile acid:Na+ symporter family; CDF, cation diffusion facilitator family; CIC, ammonium transporter family; CPA2, monovalent cation:proton antiporter-2 family; Ctr, copper transporter-2; DiT, dicarboxylate translocator family; DMT, drug/metabolite transporter; FAT, putative fatty acid transporter family; FBT, folate-biopterin transporter family; FP, ferroportin; HPPase, putative vacuolar pyrophosphatase; MCF, mitochondrial carrier family; MFS, major facilitator superfamily; MOP, multidrug/oligosaccharidyllipid/polysaccharide flippase superfamily; NCS, nucleobase:cation symporter family; PATPase, P-ATPase superfamily; PF27, PF27 family; OEP, outer envelope protein; porin-like proteins, proteins of the preprotein and amino acid transporter family; VIC, voltage gated ion channel; PLI, phospholipid importer family; SulP, sulfate permease family; Toc, translocon outer envelope of chloroplasts; Tic, translocon inner envelope of chloroplasts; MEX1, maltose excess1, a starch transporter; NTT, nucleotide transport protein; PPT, phosphoenolpyruvate phosphate translocator; TPT, triosephosphate phosphate translocator; GPT, glucose-6-phosphate phosphate translocator; XPT, pentosephosphate phosphate translocator; TGD, trigalactosyldiacylglycerol; SAMC, S-adenosyl methionine carrier. Putative transport substrates for the putative transport proteins can be looked up at www.tcdb.org/index.php.

Toc132, were both identified only in the proplastid envelope proteome sample of cauliflower. In contrast, the proteins with the lowest ratio, namely the proteins expressed more highly in the leaf, are the import complex components Tic55 and Tic62, which both could only be identified in pea leaf envelopes (Figure 2 and Supplemental Table 2). Tic32, which was only

identified in chloroplast envelopes, is of similar expression between both tissues (Figure 2).

Overall Proteome Analysis

After establishing patterns of the transport proteins for metabolites and protein import complex in the proplastid envelope, we compared the remaining proteins identified in proplastid envelopes (Supplemental Table 1) with those identified in chloroplast envelopes (Supplemental Table 2). The annotation and classification of proteins identified revealed approximately similar proportions of most classes in both proplastid and chloroplast envelopes (Figure 3A and 3B). Frequently, the proteins within one class are different (Supplemental Tables 1 and 2), such as within the enzyme class. The proplastid envelope sample did not contain sufficient amounts of enzymes of the reductive pentosephosphate pathway including Rubisco to be identified by proteomics (Supplemental Table 1). In contrast, chloroplast envelope samples contained Rubisco as a dominant protein (Ferro et al., 2003; Froehlich et al., 2003) and seven additional enzymes of the reductive pentosephosphate pathway (Supplemental Table 2) (Bräutigam et al., 2008b). Furthermore, although being a membrane proteome preparation, the proplastid sample contained ribose-phosphate pyrophosphokinase, a key enzyme of *de novo* nucleotide biosynthesis. While both envelope samples contained enzymes for pigment biosynthesis, enzymes for tocopherol biosynthesis could only be identified in chloroplast envelopes, except for VTE3, which is involved in both plastoquinone and tocopherol biosynthesis (Cheng et al., 2003) (Supplemental Table 2). The proplastid sample contained two peroxiredoxins (Dietz et al., 2002; Horling et al., 2003), enzymes for fatty acid and lipid biosynthesis, and two membrane localized proteases, FtsH11 and FtsH12. The sample also contained 65 proteins whose function was unknown and therefore could not be classified (Supplemental Table 1). Surprisingly, 16 proteins known to reside in thylakoids in chloroplasts (Peltier et al., 2000, 2002, 2004; Friso et al., 2004) were present in the proplastid envelope preparation (Figure 3A). These proteins represented only a subset of known thylakoid proteins. In the proplastid proteome, all soluble subunits of the ATP synthase were identified (i.e. alpha, beta, gamma, delta, epsilon, and F). In addition, two photosystem II subunits, PsbP-like1 and PsbS, two LHCs, two FtsH proteases, FtsH1 and FtsH8, a CAAX-type protease, the thylakoid ATP ADP carrier (Thuswaldner et al., 2007), and a cytochrome b6f complex subunit, cyt6, were identified. Publicly available microarray data indicate transcriptional down-regulation of almost all proteins of the thylakoid membranes in the shoot apex including all but the ATP ADP carrier identified by proteomics (Figure 2 and Supplemental Tables 1 and 2).

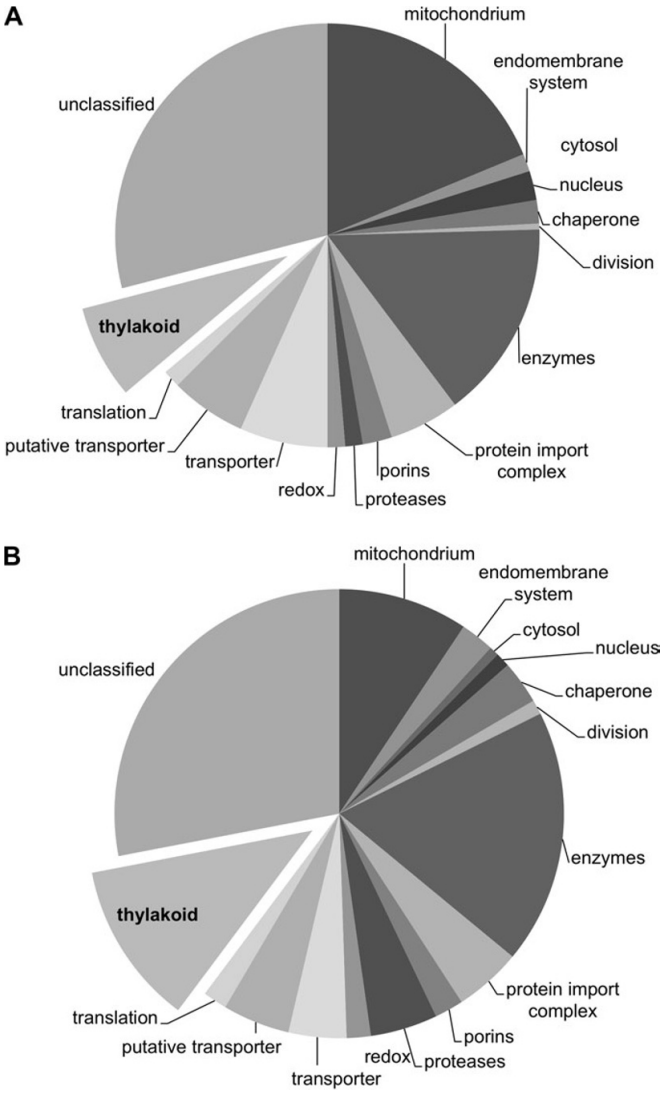

Figure 3. Overall Proteome Analysis of Proplastid (A) and Chloroplast (B) Envelope Samples.

Detailed contents of each functional class can be found in Supplemental Tables 1 and 2.

Purity of the Proteome Sample

The annotation and classification of all identified proteins (Figure 3A) revealed that about one-fifth of the detected proteins could be assigned to a location other than the plastid, mainly to the mitochondria, with only three known residents of the endomembrane system, and five residents of the nucleus. Likewise, one-fifth of the spectra assigned to proteins could be assigned to proteins of extraplastidial origin (Supplemental Table 1). In addition to extraplastidial proteins typically encountered in plastid isolations, such as histones, abundant proteins of the mitochondrial respiratory chain, and ATPases, the cauliflower proteome sample contained several unusual contaminants, such as prohibitins and an Mcm4-like protein (Supplemental Table 1), which have not been identified in other plastid envelope proteomes.

DISCUSSION

Here, we present a proteomic analysis of the plastid envelope membrane of cauliflower curd proplastids and we relate the

proteomics data to the corresponding tissue-specific transcript data in *Arabidopsis*. The proplastids used in this study were isolated according to a protocol developed by Journet and Douce (1985). These authors found that plastids isolated by this protocol retain approximately 15% of the total cellular activity for the mitochondrial marker fumarase while plastids are fourfold enriched compared to total cells (Journet and Douce, 1985). In accordance with these previous data, using spectral counting and immunoblots, we found a mitochondrial contribution to the proplastid plastid proteome sample of less than 20% (Supplemental Figure 1 and Supplemental Table 1). Hence, a significant share of the detected proteins was of mitochondrial origin. Although this share of extraplastidial proteins was considerably higher in comparison to that typically detected in chloroplast proteome studies (Bräutigam et al., 2008a, 2000b; Ferro et al., 2003; Froehlich et al., 2003; Kleffmann et al., 2004, 2007), cauliflower curd plastids nevertheless represent a suitable source for comparative analysis of proplastid and chloroplast membranes. To account for the extraplastidial contributions in our samples, the analysis was focused on proteins known to reside in plastidic membranes. The cauliflower proplastid envelope proteome was compared to the pea leaf chloroplast envelope proteome reported by Bräutigam et al. (2008b), since this study was performed on the same machine and using exactly the same proteome analysis protocols as the current study.

Proplastid and chloroplast envelopes were superficially similar, with approximately the same percentage of membrane proteins (Table 1). To increase the coverage of highly hydrophobic and moderately hydrophobic proteins, we fractionated the samples by organic solvent extraction (Seigneurin-Berny et al., 1999) in addition to analyzing a non-fractionated sample. Applying this strategy to proplastid membranes identified 49 additional proteins (Supplemental Table 1). The proteins identified in the chloroplast and the proplastid sample had a similar distribution of functional classes (Figure 3A and 3B). However, a detailed analysis of all classes' members revealed pronounced differences between the plastid types based on their role in the metabolic network of the respective cell types (Supplemental Tables 1 and 2). In non-green tissues, especially in rapidly proliferating tissues like meristematic tissue, plastids are dependent on importing precursors for biosyntheses of essential compounds that are required for cellular maintenance and growth. Chloroplasts can assimilate both inorganic nitrogen and carbon to support synthesis of complex compounds. In proplastids, both GPT and PPT serve to import carbon skeletons from the cytosol (Table 2). PPT provides PEP for aromatic amino acid biosynthesis (Knappe et al., 2003; Voll et al., 2003) and possibly—via pyruvate kinase—pyruvate for branched chain amino acid biosynthesis, for the non-mevalonate isoprenoid biosynthetic pathway, and for fatty acid biosynthesis (Andre and Benning, 2007; Andre et al., 2007) (Figure 4A). In leaf chloroplasts, PPT plays the same role, since chloroplasts do not contain a full set of glycolysis enzymes (Knappe et al., 2003; Voll et al., 2003) (Figure 4B). GPT in proplastids imports glucose-6-phosphate (Kämmerer et al., 1998), which provides building blocks via the oxidative pentose phosphate pathway (PPP) for purine and pyrimidine biosynthesis and for amino acid biosynthesis (Figure 4A). Leaf chloroplast envelopes do not contain GPT (Table 2), since they are capable of assimilating inorganic carbon to supply the reductive PPP.

The phloem sap of the *Brassicaceae Arabidopsis thaliana* contains mainly glutamate and aspartate and, to a lesser degree, glutamine, whereas the leaf phloem sap of *Brassica napus* contains mainly glutamate and glutamine and, to a lesser degree, aspartate and serine. Most amino acids needed for protein biosynthesis are not provided to sink tissues via the phloem stream (Weibull and Melin, 1990; Zhu et al., 2005) and thus have to be synthesized *de novo* in the shoot meristem to support rapid cell proliferation. Since most amino acids are synthesized in the plastid stroma, a continuous supply of glutamate to the stroma as amino group donor for aminotransferase reactions is required. In meristematic proplastids, this function is likely fulfilled by the plastidial two-translocator-system of DiT1 and DiT2 for glutamate and dicarboxylic acids, which, in leaf chloroplast, functions to export organic nitrogen in the form of glutamate from chloroplasts (Figure 4A and 4B) (Weber and Flügge, 2002; Renne et al., 2003).

The reducing power necessary to drive anabolic reactions may be provided by TPT. In leaves, TPT is the exporter of assimilated carbon via triosephosphate in exchange for inorganic phosphate (Flügge and Heldt, 1984) (Figure 4B) and it has been proposed that non-green plastids are devoid of TPT (Flügge et al., 2003). However, the TPT protein is also capable of exchanging 3-PGA and triosephosphate both *in organello* and *in vitro* (Flügge and Heldt, 1984; Loddenkötter et al., 1993), which, together with GAP-DHs, may create a reducing equivalent shuttle (Häusler et al., 2000; Heineke et al., 1991). Since the non-green proplastids do not assimilate carbon that would have to be exported from non-green plastids (Fischer and Weber, 2002), the presence of TPT indicates that it may serve as the reducing equivalent shuttle *in vivo*. This notion is further supported by the presence of plastidic GAP-DH subunits even in the membrane fraction of proplastid envelopes, indicating that GAP-DH is abundant in the proplastid stroma (Supplemental Table 1). Notably, tissues like seeds of the late torpedo stage (AtGenExpress developmental series experiment 79; Schmid et al., 2005), when triacylglycerols start to accumulate (Baud et al., 2002), have TPT expression comparable to that of the vegetative shoot apex (Figure 2), whereas later in seed development (i.e. AtGenExpress developmental series experiment 82; Schmid et al., 2005) expression declines to the level of root TPT expression. TPT thus may also supply other plastid types with reducing power, which complements the reducing power supplied by glucose-6-phosphate via the GPT (Wakao et al., 2008; Wakao and Benning, 2005).

The anabolic reactions require not only reducing power, but also energy input to proceed and the proplastid envelope contains both isoforms of the plastidic ATP/ADP carrier, NTT. The absence of photophosphorylation in non-green tissues further

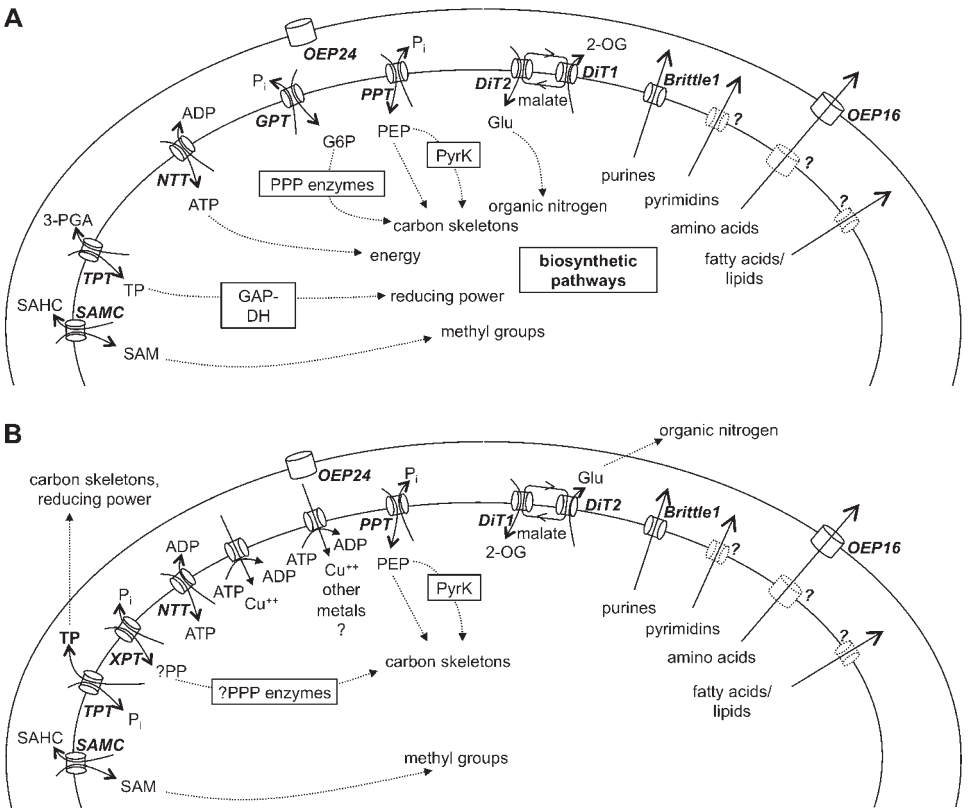

Figure 4. Suggested Transport Connections across the Proplastid (A) and Chloroplast (B) Envelope Based on Protein Presence Detected in Proteomics Experiments.

SAMC, S-adenosylmethionine carrier; SAM, S-adenosylmethionine; SAHC, S-adenosylhomocysteine; TPT, triosephosphate phosphate translocator; TP, triosephosphate; 3-PGA, phosphoglycerate; GAP-DH, glycerinaldehyde dehydrogenase; NTT, nucleotide transporter; GPT, glucose-6-phosphate phosphate translocator; P_i, inorganic phosphate; G6P, glucose-6-phosphate; PPP, pentosephosphate pathway; PPT, phosphoenolpyruvate phosphate translocator; PEP, phosphoenolpyruvate; PyrK, pyruvate kinase; DiT, dicarboxylate translocator; Glu, glutamate; 2-OG, 2-oxoglutarate; OEP, outer envelope protein; XPT, pentosephosphate phosphate translocator; PP, pentosephosphate.

confirms previous evidence indicating that these transporters work as importers of ATP into plastids (Reinhold et al., 2007; Reiser et al., 2004) (Figure 4A). Chloroplasts are dependent on low levels of nocturnal ATP import (Reinhold et al., 2007) whereas heterotrophic plastids are entirely dependent on ATP import from the cytosol, which may explain the high expression level of NTT in the meristematic tissue of the shoot apex (Table 2). In meristematic proplastids, both purines and pyrimidines and amino acids have to be exported to the cytosol (Zrenner et al., 2006). The purine exporter is Brittle1-like, the plastidic adenine nucleotide uniporter (Kirchberger et al., 2008). Although expression via GUS staining was not reported in the shoot apical meristem (Kirchberger et al., 2008), publicly available microarray data supported the identification of Brittle1-like in proplastid envelopes, which has not been detected previously in plastid proteome projects (Table 2). Like purines, pyrimidines need to be exported, as the plastids are the sole site of their biosynthesis *in planta* (Zrenner et al., 2006). The exporter remains unknown and it may be one of the transport proteins of unknown function identified in this study (Table 2 and Supplemental Table 1), which are also highly expressed in the meristematic tissue of the shoot apex compared to leaves (Figure 2), such as the MCF member At5g64970. Proteins involved in the export of amino acids from plastids have only been identified for the passage through the outer envelope (Pohlmeyer et al., 1997), while the route for amino acid transport across the inner envelope is still unknown. Although label-free quantitative proteomics is not possible for samples for which no species-specific database is available, it was noteworthy that the outer envelope protein for amino acid transport, OEP16 (Pohlmeyer et al., 1997), was among the proteins identified with the highest number of spectra (Supplemental Table 1), as were some related proteins of the Tim17/Tim22/Tim23 family (Murcha et al., 2007) (Supplemental Table 1). It is tempting to hypothesize that the inner envelope transporter for amino acids might also belong to the class of broad specificity porin-like proteins. Passage of the sugar phosphates, dicarboxylates, charged amino acids, and ATP through the outer envelope may be mediated by the outer envelope protein, OEP 24 (Pohlmeyer et al., 1998) (Figure 4A and B).

Several proteins, which are similar to known transport proteins, were identified in the proplastid proteome sample (Table 2). This includes proteins of the sodium bile acid

symporter family. Sodium-coupled transport of anions across a plastid envelope has been demonstrated (Aoki and Kanai, 1997; Aoki et al., 1992). However, it remains unresolved how a sodium gradient may be energized. The proplastid envelope sample contained putative sodium:phosphate and sodium:proton transport proteins (Table 2), which suggests that different ion gradients can be generated by coupling them to the proton gradient of the envelope.

Comparing the known and putative transport proteins identified in this study with those identified in other plastid proteome projects showed major differences (Table 2). However, except for the plastid envelope studies undertaken in maize (Bräutigam et al., 2008a) and pea (Bräutigam et al., 2008b), the difference may be due to differences in sample isolation, treatment, and protein identification. For example, the proplastid proteome study of BY-2 cells (Baginsky et al., 2004) identified only four of 30 transport proteins identified in this study. This is most likely due to the fact that the focus of this study was on total plastid proteins, thereby leading to abundant soluble proteins masking some of the harder to ionize membrane proteins (Eichacker et al., 2004), rather than differences in the proplastids themselves.

The analyses of expression patterns for known and putative envelope transport proteins show different expression patterns for the proteins identified. Compared to leaves, the expression of TPT is down-regulated in the shoot apex (Figure 2), questioning the presence of TPT in the shoot apical meristem. Proteomics, however, indicated protein presence, which led to a novel hypothesis about the functional roles of GPT and TPT in the meristematic tissue of the shoot apex. Although microarray likewise reports a down-regulation of the DiT1/DiT2 two-translocator system in the shoot apex (Figure 2), the proteomic analysis nevertheless clearly detects both proteins in this tissue (Table 2). The expression of Brittle1-like in the shoot apical meristem was not detected by promoter:GUS fusion analysis (Kirchberger et al., 2008); proteomic analysis, however, revealed Brittle1-like as a proplastid envelope protein. In this case, the publicly available microarray data indicate that Brittle1-like is highly expressed in the shoot apex, underscoring its role in the meristematic tissue (Figure 2). In contrast to microarrays, where analysis and hypothesis development are frequently dependent on comparing two or more conditions, the proteomics experiments state the absolute presence of a protein, thereby allowing hypotheses to be based on protein presence.

A difference in the metabolic role between chloroplasts and proplastids was apparent not only in the permeome, but also in the proteins of the protein import complex, where some subunits display a tissue specific expression pattern (Figure 2 and Supplemental Tables 1 and 2). Toc120 and Toc132 are alternative import receptors to Toc159 (Jackson-Constan and Keegstra, 2001; Ivanova et al., 2004; Kubis et al., 2004). If both are knocked out, plants are severely compromised in their ability to establish growth, contain almost no chlorophyll, and have damaged chloroplasts (Kubis et al., 2004). It has been proposed that Toc132 and Toc120 are essential for the import of housekeeping proteins into plastids, whereas Toc159 is specialized for the import of proteins involved in photosynthesis (Ivanova et al., 2004; Kubis et al., 2004; Kessler and Schnell, 2006), although the distinction of imported products is not absolute (Kubis et al., 2004). Since, to date, neither Toc132 nor Toc120 has been identified by proteomics in either chloroplast or chloroplast envelope studies (Supplemental Table 1), it may be concluded that the protein levels of both proteins are rather low in leaves, thereby precluding identification by proteomics, whereas their higher expression level in meristematic tissue (Figure 2) permits proteomic identification (Supplemental Table 1). Toc132 and Toc120 can also be identified by phosphoproteomics from cultured cells (Sugiyama et al., 2008). Toc75, Tic110, Tic20, Tic22, and Tic40 were identified in proplastids as well as chloroplast envelopes in line with their housekeeping function (Figure 2) (Schnell, 2000; Jarvis and Robinson, 2004; Soll and Schleiff, 2004). The members of the redox-sensing complex, however, Tic32, Tic62, and Tic55 (Küchler et al., 2002; Soll and Schleiff, 2004; Stengel et al., 2007, 2008) could not be identified in proplastid envelope proteomics. This may be due either to a lower expression level, for example of Tic55 and Tic62 (Figure 2), or to a lack of membrane association of any of the components. Unlike members of the small molecule permeome, the proteomic identifications of TIC/TOC components map well to the expression pattern of the respective genes (Figure 2 and Supplemental Tables 1 and 2).

As in other plastid envelope proteome projects, a number of apparently soluble proteins were also identified. These may represent proteins associated with the membranes, such as enzymes involved in fatty acid and lipid biosynthesis, or proteins that are rather abundant in the tissue.

In the proplastid envelope proteome, a subset of known thylakoid proteins was identified. Due to their high abundance, thylakoid membrane proteins inevitably are detected in envelope proteome analyses across several species analyzed (Ferro et al., 2003; Froehlich et al., 2003; Bräutigam et al., 2008a, 2008b). Compared to the proplastid membrane proteome sample, however, the spectrum in chloroplasts is much broader and also includes many subunits of the photosystems and light-harvesting complex proteins (Supplemental Table 2) (see, e.g. Bräutigam et al., 2008b). The only proteins abundant enough to be identified by proteomics in cauliflower proplastid envelopes are components of the ATP synthase and two PS II components, whereas no PS I components could be detected. One of the two detected PSII components, PsbS, is involved in redox protection by playing a key role in non-photochemical quenching (Li et al., 2000) via its role in the association/dissociation of the PSII antenna complex (Betterle et al., 2009). The other detected PS II component, PsbPL-1, is involved in PS II assembly and stability (Ishihara et al., 2007). Both proteins accumulate in etioplasts compared to chloroplasts (Kanervo et al., 2008). Only one protein of the light-harvesting complex could be detected, although these proteins represent the most

abundant thylakoid-derived contaminants of chloroplast envelope samples isolated from leaves. The absence of enzymes of the reductive PPP and the limitation of thylakoid resident proteins to functions not directly related to photosynthetic electron transport strongly indicate the absence of carbon fixation in proplastids, as already indicated by the absence of a developed thylakoid system and the absence of chlorophyll (Journet and Douce, 1985). The subset of proteins identified strikingly mirrors that identified in rice etioplasts (see Supplemental Table of von Zychlinski et al., 2005). Analysis of the protein complexes present in different stages of greening also shows that the ATP synthase, the cytochrome b6f complex, and an FtsH complex accumulate in etioplasts (Kanervo et al., 2008; Lonosky et al., 2004). Both photosystem II subunits identified, PsbS and a PsbP, are also already present in etioplasts (Kanervo et al., 2008). Analysis of proplastid envelopes demonstrates that the accumulation of these proteins predates plastid differentiation into etioplasts (Supplemental Table 1). This pattern cannot be tied to changes in transcript accumulation (Figure 2 and Supplemental Table 1) and is likely regulated at the posttranscriptional level (Kanervo et al., 2008). Detection of the thylakoid ATP ADP carrier (Thuswaldner et al., 2007) has not been reported in either study. Unlike any of the other thylakoid proteins identified, the carrier is also highly expressed in the meristematic tissue of the shoot apex.

On one hand, the proplastids, like etioplasts, appear poised to differentiate; they are equipped to both dissipate the proton gradient through the ATP synthase and handle the redox stress generated during photosynthetic electron transfer (Kanervo et al., 2008). On the other hand, ATP synthase may play specific roles during development. ATP synthase is present in differentially post-translationally modified isoforms during greening of etioplasts, which led to speculation about different functions (Lonosky et al., 2004). Possibly, ATP synthase is needed to produce a proton gradient across the internal membrane system of proplastids. Like plastids themselves, the internal membrane system has never been reported to appear *de novo* in dividing plastids but is extended from the internal membrane system already present. Of the four known protein import pathways, one is known to be dependent on a proton gradient at least *in vitro* (reviewed in Aldridge et al., 2009; Cline and Dabney-Smith, 2008; Schünemann, 2007), although this notion has recently been controversially discussed (Di Cola et al., 2005; Finazzi et al., 2003; Theg et al., 2005). Both the cpSec and the cpSRP protein import pathways are at least stimulated by the presence of a proton gradient (reviewed in Aldridge et al., 2009; Cline and Dabney-Smith, 2008; Schünemann, 2007). Possibly, the presence of ATPase in internal membrane systems of nongreen precursors for chloroplasts, proplastids, and etioplasts is needed to build a proton gradient for protein import into and differentiation of the internal membrane system into thylakoids. In addition, sequestering protons within the thylakoid lumen generates a proton gradient across the plastid envelope membranes, which drives proton-coupled transport processes.

Conclusions

Proteomics of the proplastid membrane system identifies proplastid-specific patterns. The transport proteins of the envelopes are specifically adapted to supply the proliferating cells of the shoot apical meristem with the necessary building blocks and the protein import complex is geared to import proteins involved in metabolism rather than photosynthesis. Overall proteome analysis indicates proplastids are not only equipped to support cellular growth, but also adapted to differentiate upon light exposure. Cauliflower curd thus represents a promising model to study plastid differentiation. Whereas shaded cauliflower curd contains proplastids, differentiation into chloroplasts can be induced by exposure to light, and differentiation into chromoplasts can be studied in the orange variety of cauliflower, thus permitting the study of three different plastid types in the same system.

METHODS

Isolation and Treatment of Proplastid Envelope Membranes

Cauliflower curd plastids were isolated according to Journet and Douce (1985). The plastids were lysed and plastid envelopes were prepared as described in Douce and Joyard (1979) and Keegstra and Yousif (1986). The envelope membranes were separated into chloroform/methanol (1:1, v/v) soluble and insoluble fractions by diluting a re-suspended pellet of envelope membranes with 10 volumes of solvent (Seigneurin-Berny et al., 1999). After incubation on ice for 20 min, phases were separated by centrifugation (20 000 g; 20 min). The chloroform-soluble fraction was then transferred to a fresh tube, whereas the insoluble fraction was recovered as a pellet after removal of the supernatant. Both fractions were dried, washed with hexane to remove lipids, carefully dried again at room temperature, and dissolved in SDS–PAGE loading buffer. All fractions were separated by 12.5% SDS–PAGE.

Protein Identification

Gels were briefly stained with Coomassie Brilliant Blue and then cut into 10 equally sized slices. Proteins within each slice were modified and digested with trypsin as described by Shevchenko et al. (1996). After extraction, the peptides were loaded by a Waters nanoAcquity Sample Manager onto a Waters Symmetry C18 peptide trap (5 μm, 180 μm $\times$ 20 mm) at a flow rate of 4 μL min^{-1} in 2% Acetonitrile/0.1%Formic Acid for 5 min. Separation of peptides occurred on a Waters BEH C18 nanoAcquity column (1.7 μm, 100 μm $\times$ 100 mm) for 90 min and the peptides were injected into a Thermo-Electron LTQ-FTICR mass spectrometer with a flow rate of

300 nL min^{-1} (Buffer A = 99.9% Water/0.1% Formic Acid, Buffer B = 99.9% Acetonitrile/0.1% Formic Acid: gradient of 5% B to 40% B from 0 to 63 min, 40% B to 90% B from 63 to 71 min and 5% B from 71 to 90 min). The top 10 ions of each survey scan (resolution 50 000) were automatically dissociated by low-energy collision. The resulting MS/MS spectra were converted to a peak list by the BioWorks Browser version v3.2. Peptides were identified by comparing all mass spectra libraries to a sequence database from *Arabidopsis thaliana*, TAIR 8 (Swarbreck et al., 2008) and to a sequence database from *Brassica napus* (BnGI, ftp://occams.dfci.harvard.edu/pub/bio/tgi/data/Brassica_napus/) using the Mascot search algorithm v 2.2 (www.matrixscience.com). Oxidation of methionine was permitted and carbamidomethylcysteine was set as fixed peptide modifications. Two missed tryptic sites were allowed and the MS/MS tolerance was set to 0.8 kDa and the peptide tolerance to +/– 10 ppm. The results of the Mascot search algorithm for each database were loaded into Scaffold® and analyzed with peptide and protein prophet algorithms (Keller et al., 2002). Parameters were set to 99% confidence for protein identification, 95% confidence for peptide identification, and at least two peptides identified for each protein. When multiple matches were reported, the matches were analyzed manually and lower-scoring matches were discarded. Results were exported to MS Excel for further analysis and combined into one table (Supplemental Table 1). Original data can be downloaded from the PRIDE repository for proteome experiments (Martens et al., 2005) as PRIDE Experiment Accession # 9755.

Protein Analysis

Annotation and classification of proteins were assigned based on information in TAIR8 (Swarbreck et al., 2008), ARAMEMNON (Schwacke et al., 2003), and manual curation of the literature. The predicted location, the number of transmembrane helices, and the prediction of betafold structures were retrieved from TAIR. Comparison to data from other proteome projects was conducted using plprot (Kleffmann et al., 2006) and manual comparison to supplemental data tables (Ferro et al., 2002; Froehlich et al., 2003; Bräutigam et al., 2008a; Zybailov et al., 2008), except for specific comparison to a chloroplast envelope proteome dataset generated with identical protocols. Data from these pea chloroplast envelopes described in Bräutigam et al. (2008b) were extracted from the public repository PRIDE and reanalyzed according to the same specifications (Supplemental Table 2).

Gene Expression Analysis

For each protein identified in proteomics and for proteins of a virtual plastid envelope based on metaproteome analysis (Weber et al., 2005), the gene expression data were extracted from the AtGenExpress Developmental series (Schmid et al., 2005). The virtual plastid envelope can be loaded as a 'mapping' file for the Mapman software (Thimm et al., 2004) and displayed onto the virtual plastid 'pathway' (Supplemental Files 1 and 2). The shoot apex expression level and the leaf expression level were calculated as the arithmetic mean from experiments ATGE_6 and ATGE_15. The ratio of expression was calculated as the $\log_2$ of the quotient expression (shoot apex) divided by expression (leaf). The table can be loaded as a Mapman 'experiment' (Supplemental File 3).

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

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