Chloroplast Proteins without Cleavable Transit Peptides: Rare Exceptions or a Major Constituent of the Chloroplast Proteome?

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ABSTRACT Most chloroplast proteins (cp proteins) are nucleus-encoded, synthesized on cytosolic ribosomes as precursor proteins containing a presequence (cTP), and post-translationally imported via the Tic/Toc complex into the organelle, where the cTP is removed. Only a few unambiguous instances of cp proteins that do not require cTPs (non-canonical cp proteins) have been reported so far. However, the survey of data from large-scale proteomic studies presented here suggests that the fraction of such proteins in the total cp proteome might be as large as ;30%. To explore this discrepancy, we chose a representative set of 28 putative non-canonical cp proteins, and used in vitro import and Red Fluorescent Protein (RFP)-fusion assays to determine their sub-cellular destinations. Four proteins, including embryo defective 1211, glycolate oxidase 2, protein disulfide isomerase-like protein (PDIl), and a putative glutathione S-transferase, could be unambiguously assigned to the chloroplast. Several others ('potential cp proteins') were found to be imported into chloroplasts in vitro, but failed to localize to the organelle when RFP was fused to their C-terminal ends. Extrapolations suggest that the fraction of cp proteins that enter the inner compartments of the organelle, although they lack a cTP, might be as large as 11.4% of the total cp proteome. Our data also support the idea that cytosolic proteins that associate with the cp outer membrane might account for false positive cp proteins obtained in earlier studies.

Key words: Chloroplast biology; mitochondria; organelle biogenesis/function; protein targeting.

INTRODUCTION

The vast majority of chloroplast (cp) proteins is encoded in the nucleus and synthesized on cytosolic ribosomes as precursor proteins (preproteins) (Jarvis, 2008). Each preprotein generally possesses an N-terminal extension (or 'presequence')—the cp transit peptide (cTP)—which post-translationally directs it to the organelle and is removed upon arrival in the organelle (Soll and Schleiff, 2004). This import process is mediated by the sequential action of two multi-protein complexes Toc and Tic (Translocon at the Outer/Inner envelope membrane of Chloroplasts), which permit passage across the two envelope membranes (Oreb et al., 2008; Benz et al., 2009; Sommer and Schleiff, 2009). Based on the bioinformatic identification of genes that code for cTP sequences in Arabidopsis thaliana, the cp proteome has been estimated to contain between 2000 and 3600 different cTP-bearing proteins (Abdallah et al., 2000; Peltier et al., 2002; Leister, 2003; Richly and Leister, 2004).

However, not all bona fide cp proteins possess a cleavable presequence. Most proteins of the outer cp envelope membrane clearly lack a cTP (Soll and Schleiff, 2004; Jarvis, 2008). Instead, they are directed to the outer cp envelope by intrinsic targeting information. For many years, all proteins destined for internal cp compartments were thought to possess a cTP, and to engage the Toc/Tic machinery. However, new studies have revealed the existence of several proteins that lack cleavable presequences but reach internal cp compartments nevertheless. The first evidence for such 'non-canonical' cp proteins was provided by studies on the Arabidopsis envelope proteome, which led to the identification of the ceQORH (chloroplast envelope Quinone Oxidoreductase Homologue) protein (Miras et al., 2002; Ferro et al., 2003). Intriguingly, despite its

lack of a cTP, ceQORH is found associated with the inner envelope. Instead of the N-terminal segment, an internal sequence stretch of ;40 amino acids controls cp localization of ceQORH (Miras et al., 2002). Moreover, although targeting of ceQORH requires a proteinaceous import apparatus and ATP, the process is not mediated by the standard Toc/Tic machinery (Miras et al., 2007). More recently, Tic32/IEP32 was identified as another inner cp envelope protein without a cTP (Nada and Soll, 2004). Tic32/IEP32 is imported into chloroplasts at low ATP concentrations and without the assistance of key components of the Toc machinery, but—in contrast to ceQORH—the targeting information for Tic32/IEP32 resides at its extreme N-terminus.

The existence of a second class of non-canonical cp proteins was initially suggested on the basis of cp proteome studies. In particular, the identification of a fraction of cp proteins with predicted signal peptides (SP) for ER translocation (Kleffmann et al., 2004) suggested the existence of a novel pathway for cp protein import. That the ER and the outer cp envelope membrane interact both physically and metabolically (in lipid biosynthesis) has been known for many years, and trafficking of cp proteins through the endomembrane system is well documented in organisms that have complex plastids, including many algae and apicomplexan parasites (reviewed in Jarvis, 2008). Cp proteins in species with complex plastids typically have a bipartite targeting sequence, composed of an N-terminal SP fused to a more or less standard cTP (Kleine et al., 2008). The SP directs the precursor into the ER and is removed there, and the protein then passes along the secretory pathway until it arrives at the plastid, at which point the cTP mediates cp import in the usual fashion (Nassoury and Morse, 2005). A recent study provided evidence that an ER-dependent targeting pathway for cp proteins actually exists in flowering plants also (Villarejo et al., 2005). Thus, the Arabidopsis carbonic anhydrase 1 (CAH1) protein was found to localize in the cp stroma, although it was predicted to have an SP. Multiple lines of evidence suggested that CAH1 is imported into chloroplasts via the ER: (1) CAH1 could not be imported directly by isolated chloroplasts, but was taken up co-translationally by pancreatic microsomes and concomitantly processed to its mature size; (2) stromal CAH1 is glycosylated with glucans that are only added in the Golgi; and (3) brefeldin A (which interferes with Golgi-mediated vesicle traffic) arrests transport of CAH1 within the endomembrane system (Villarejo et al., 2005). More recently, nucleotide pyrophosphatase/phosphodiesterase 1 (NPP1) was reported to follow a similar ER-dependent cp targeting pathway (Nanjo et al., 2006). Based on its similarities to cp protein transport in primitive organisms, the CAH1 pathway might represent an ancestral co-translational targeting mechanism that arose prior to the evolution of the now dominant post-translational Toc/Tic system (Villarejo et al., 2005).

A third possible non-canonical cp import pathway has been proposed for NADPH:protochlorophyllide oxidoreductase (POR). Of the two main isoforms of POR, POR-B is light-stable and is translocated through the standard Toc/Tic import apparatus, whereas POR-A is light-labile and has been reported to utilize a substrate-dependent import mechanism that is distinct from the Toc/Tic pathway. Specifically, POR-A import is proposed to occur only when the preprotein has bound its substrate, protochlorophyllide (Reinbothe et al., 1995, 1997). The non-canonical POR-A import mechanism is supported by in vivo data (Kim and Apel, 2004; Kim et al., 2005), and components of a putative import apparatus have been identified and studied (Reinbothe et al., 2005; Pollmann et al., 2007; Schemenewitz et al., 2007; Reinbothe et al., 2008). However, the existence of the pathway has been strongly disputed, and it has been suggested that the reported substrate dependency of POR-A import is an experimental artifact (reviewed in Jarvis, 2008).

Considering all the evidence, it appears clear that at least two classes of non-canonical cp proteins exist: the cp inner-envelope type and the ER-dependent type. However, the extent to which non-cTP cp proteins contribute to the cp proteome is not at all clear. Do the few cases described above represent rare exceptions or are they only the tip of the iceberg? To help answer this question, we have collected and analyzed proteomic data on putative non-cTP cp proteins, and subjected a representative set of candidates to experimental tests to determine their actual sub-cellular locations.

RESULTS

Survey of Chloroplast Proteomic Studies and the Non-Canonical Chloroplast Proteins Identified Therein

Over the past several years, the chloroplast proteome (or various subfractions of it) have been the target of a number of systematic studies (Table 1). When the results of these studies are pooled, one finds that 1449 different nucleus-encoded proteins have so far been assigned to the chloroplast by at least one investigation (row 'Total' in Table 1, and Supplemental Table 1). Removal of 57 obvious contaminants or proteins most likely associated with the cp outer-envelope, such as subunits of cytoplasmic ribosomes, leaves us with a set of 1392 proteins referred to as 'tentative cp proteins' (row 'Final total' in Table 1). A subset of 445 (or 32%) of these represent non-canonical cp proteins, because they lack a predicted cTP. Interestingly, the relative numbers of such proteins vary depending on the subcompartments of the chloroplast considered. They account for between 23 and 27% of the sets found in preparations of cp envelopes (Ferro et al., 2003; Froehlich et al., 2003). This is perhaps not unexpected, because various outer-envelope proteins have been shown to insert into the membrane without requiring a cTP (see Introduction). In the studies that set out to identify proteins of the internal compartments of chloroplast–thylakoids (Peltier et al., 2002; Schubert et al., 2002; Friso et al., 2004; Peltier et al., 2004; Giacomelli et al., 2006) and stroma (Peltier et al., 2006; Rutschow et al., 2008), the fraction of non-cTP proteins is markedly lower, ranging from 4 to 10% for thylakoids and 12 to 13% for the stroma. In the two studies that directly analyzed total chloroplasts,

Table 1. Fraction of Non-Canonical Chloroplast Proteins Identified in Proteomic Studies of Chloroplast Proteins.

Designation	cp subcompartment	Number of nucleus-encoded proteins				Reference
		Total	Without predicted cTP*	With mTP	With SP	
1_env	envelope	351	81 (23%)	22 (6%)	5 (1%)	Froehlich et al., 2003
2_env	envelope	125	34 (27%)	3 (2%)	0	Ferro et al., 2003
3_tot	cp total	485	92 (19%)	28# (6%)	24# (5%)	Kleffmann et al., 2004
4_tot	cp total	916	128 (14%)	51# (6%)	6# (1%)	Zybailov et al., 2008
5_thy	thy membrane	179	18 (10%)	4* (2%)	2 (1%)	Friso et al., 2004
6_thy	thy membrane	221	17 (8%)	12 (5%)	5 (2%)	Peltier et al., 2004
7_thy	thy lumen and peripheral proteins	65	3 (5%)	0	0	Peltier et al., 2002
8_thy	thy lumen and peripheral proteins	95	9 (9%)	1 (1%)	3 (3%)	Giacomelli et al., 2006
9_thy	thy lumen	47	2 (4%)	0	0	Schubert et al., 2002
10_str	stroma	234	29 (12%)	7* (3%)	1* (0%)	Peltier et al., 2006
11_str	stroma	263	33 (13%)	6 (2%)	3 (1%)	Rutschow et al., 2008
Total		1449	504 (35%)	131 (9%)	73 (5%)	This study
Final total**		1392	445 (32%)	117 (8%)	73 (5%)	This study

* Predicted by TargetP and/or Predotar.
** After removal of obvious contaminants or proteins most likely associated with the cp outer-envelope, such as 80S-type ribosome subunits.
# Number cited in the relevant original publication. cp, chloroplast; thy, thylakoid; str, stroma; tot, total.

between 14 and 19% of the proteins assigned to the chloroplast were found to lack a predicted cTP.

Further analysis of the N-terminal sequences of the 445 tentative non-canonical cp proteins revealed that 117 possess a mitochondrial targeting peptide (mTP) (Table 1 and Supplemental Table 1). This suggests that these proteins actually contain N-terminal transit peptides, which (1) either act as cTPs but could not be recognized as such because their sequences did not match the known sequence pattern used for predictor development, or (2) represent transit peptides that target the proteins to both chloroplasts and mitochondria, or (3) only target the proteins to the mitochondria and are therefore contaminants of mitochondrial origin. A second, smaller set of 73 proteins contained SPs (Table 1 and Supplemental Table 1) and are candidates for the ER-dependent cp targeting pathway, for which CAH1 and NPP1 serve as prototypes (see Introduction).

Defining a Test Set for Experimental Verification of Sub-Cellular Location of Non-Canonical Chloroplast Proteins

In principle, the large number of non-cTP cp proteins identified by proteomic analyses of chloroplasts can be accounted for in two ways: either (1) the fraction of non-canonical cp proteins is indeed much larger than previous estimates based on conventional single-protein studies; or (2) proteomic chloroplast analyses, particularly those devoted to total chloroplast proteomes (Kleffmann et al., 2004; Zybailov et al., 2008), have been affected by contamination with non-cp proteins.

In order to distinguish between these alternatives, we selected a subset of the 445 tentative non-canonical cp proteins for direct experimental tests to define their sub-cellular location. The main criterion for their selection was a robust prediction that they really lack cTPs. Because the maximum accuracy of cTP prediction is achieved by combining several prediction programs (Richly and Leister, 2004), nine different algorithms for cTP prediction were employed. Only proteins that were predicted to lack a cTP by at least seven of the nine predictors were selected. In addition, proteins were preferred that had been identified by several independent studies of the cp proteome. Taking these criteria together, a test set of 25 tentative non-canonical cp proteins was selected, of which 18 had been found by at least two independent cp proteomics studies (Table 2). Moreover, within this set, the proteins At2g45740, At3g14210, At3g54960, and At4g39730 were predicted to contain an SP.

In addition, we checked published cp proteins identified by immunolocalization for members that lack cTPs. Among them were At3g47070 (TSP9) and At5g42960 (OEP24.2) (Table 3), which had also been identified by proteomic studies (see Table 2). Furthermore, the three thylakoid-associated kinases (TAKs) were included, which have been reported to localize to chloroplasts but lack cTPs (Table 3). Taking the two groups listed in Tables 2 and 3 together, the final test set chosen comprises 28 tentative non-cTP cp proteins.

Several Tentative Non-Canonical Chloroplast Proteins Can Be Directly Demonstrated to Localize in the Organelle

Sub-Cellular Localization by Tracking of RFP Fusions and In Vitro Import Assays

To experimentally determine the sub-cellular location of the members of the 28-protein test set, two assays were employed. First, their full-length ORFs were fused to the sequence encoding the Red Fluorescent Protein (RFP) DsRED (Jach et al., 2001) and transfected into Arabidopsis protoplasts. In this RFP-fusion

Table 2. Tentative Non-Canonical Chloroplast Proteins Identified in Proteomics Studies and Selected for Experimental Determination of their Sub-Cellular Location.

Protein	Description	Number of proteomic studies*	cp proteomic studies according to Table 1	Non-cp prediction**
At1g16790	Ribosomal protein-related	3/3	1_env, 4_tot, 6_thy	9
At1g22450	Putative cytochrome c oxidase subunit 6b (COX6B)	6/2	3_tot, 5_thy	8
At1g35720	Annexin 1 (ANN1)	18/3	3_tot, 7_thy, 10_str	9
At1g64520	Putative 26S proteasome regulatory subunit (RPN12)	1/1	2_env	8
At1g78380	Glutathione S-transferase	17/2	4_tot, 10_str	9
At2g11910	Expressed protein	1/1	1_env	9
At2g45740	Peroxisomal biogenesis factor 11 family protein (PEX11)	4/2	3_tot, 4_tot	9
At3g02520	14–3–3 protein GF14 nu (GRF7)	6/1	3_tot	9
At3g14210	Epithiospecifier modifier 1 (ESM1)	13/3	1_env, 3_tot, 4_tot,	8
At3g14420	Glycolate oxidase 2, GOX2	11/3	3_tot, 4_tot, 11_str	9
At3g16640	Translationally controlled tumor protein (TCTP)	15/2	4_tot, 7_thy,	9
At3g22520	Unknown protein	4/4	1_env, 3_tot, 4_tot, 11_str	9
At3g47070	TSP9	4/4	1_env; 4_tot; 5_thy; 6_thy	9
At3g52930	Putative fructose bisphosphate aldolase	22/1	3_tot	8
At3g54960	Protein disulfide isomerase-like protein (PDII)	3/1	3_tot	9
At4g02520	Putative glutathione S-transferase	19/4	3_tot, 4_tot, 10_str, 11_str	9
At4g09000	14–3–3-like protein GRF1 chi	15/2	3_tot, 4_tot	8
At4g25100	Fe-superoxide dismutase	16/5	3_tot, 4_tot, 2_env, 7_thy, 10_str	7
At4g25210	Transcription regulator	2/1	3_tot	8
At4g35000	Ascorbate peroxidase (APX3)	13/3	1_env, 2_env, 4_tot,	9
At4g39730	Lipid-associated family protein	7/4	3_tot, 5_thy, 6_thy, 8_thy	8
At5g22640	Embryo defective 1211 (EMB1211)	3/3	1_env, 4_tot, 6_thy	9
At5g42960	OEP24.2	4/3	1_env, 3_tot, 4_tot	7
At5g42980	Thioredoxin H (ATH3)	14/2	4_tot, 10_str	9
At5g51540	Metalloendopeptidase	2/2	1_env, 6_thy	7

* Total number of proteomic studies in which the protein was identified and how many of these assigned it to the chloroplast, according to The Plastid Proteome Database (PPDB; http://ppdb.tc.cornell.edu);
** number of algorithms that predict a non-cp location, namely absence of a cTP. In all, nine different predictors were employed: TargetP, Predotar, ChloroP, Wolfpsort, iPSORT, PSORT; PCLR, BaCelLo, ProteinProwler.

Table 3. Non-Canonical Chloroplast Proteins Identified by Conventional Biochemical Analyses and Selected for Experimental Determination of their Sub-Cellular Location.

Protein	Description	Non-cp prediction	Reference
At4g02630	Thylakoid-associated kinase 1 (TAK1)	6	Snyders and Kohorn, 1999
At1g01540	Thylakoid-associated kinase 2 (TAK2)	6	Snyders and Kohorn, 1999
At4g01330	Thylakoid-associated kinase 3 (TAK3)	7	Snyders and Kohorn, 1999
At3g47070	Thylakoid soluble phosphoprotein of 9 kDa (TSP9)	9	Carlberg et al., 2003
At5g42960	24-kD chloroplastic outer-envelope membrane protein (OEP24.2)	7	Schleiff et al., 2003

assay, the reporter is fused to the C-terminal end of the test protein, and comparison of the position of the RFP signal with the distribution of chlorophyll autofluorescence allows one to monitor the sub-cellular location of the tentative cp proteins. As a complementary approach, we chose an in vitro import assay. The proteins were radiolabeled by in vitro transcription and translation, and incubated with isolated pea chloroplasts. The chloroplasts were then recovered by centrifugation through Percoll, and incubated with thermolysin to remove non-imported proteins. Samples taken before and after thermolysin treatment, as well as aliquots of the translation products, were then subjected to SDS–PAGE and autoradiography. In the in vitro import assay, detection of the protein prior to thermolysin treatment indicates that the protein has attached to the organelle; if the signal persists after thermolysin treatment, one infers that import has occurred. Moreover, the

presence of an additional smaller band is characteristic for post-import cleavage of the cTP. In addition, after successful import reactions, chloroplasts were first lysed by addition of detergent and then treated with thermolysin to investigate whether import signals are truly due to localization of the proteins inside the chloroplasts and not to protection by other proteins. Results of the RFP-fusion and in vitro import assays are shown for all 10 proteins identified as confirmed or potential non-canonical cp proteins (Figure 1), as well as a representative set of tentative non-cTP cp proteins for which an intra-organellar location could not be confirmed (Figure 2).

Identification of Four Non-Canonical cp Proteins

For At3g14420, At3g54960, At4g02520, and At5g22640, a cp location was experimentally demonstrated (Table 4 and Figure 1A). The RFP-fusion assay indicated that these proteins are located either in chloroplasts alone (At5g22640) or in chloroplasts and other compartments (the other three proteins). In the in vitro import assay, all four proteins were found to be imported into chloroplasts, but without processing of the presequence, as expected for proteins that lack a cleavable cTP.

Interestingly, At3g14420 (isoform 2 of glycolate oxidase, GOX2) is known to be located in peroxisomes (Reumann et al., 2007). In peroxisomes, glycolate oxidase is involved in the photorespiratory C_2 cycle. Its substrate, glycolate, is formed in chloroplasts and diffuses into the matrix of peroxisomes, where it is oxidized to glyoxylate, with concomitant production of H_2O_2 (reviewed in Reumann and Weber, 2006). Because there is no evidence that glycolate oxidation occurs in chloroplasts, it is unlikely that GOX2 accumulates to physiologically relevant levels in the organelles. However, it is conceivable that GOX2 might catalyze a different reaction in chloroplasts.

Identification of Six Potential Non-Canonical cp Proteins

The proteins At1g22450, At3g47070, At4g02630, At4g09000, At4g35000, and At5g42960 failed to qualify as cp proteins in the RFP-fusion assay, but clearly exhibited a cp location in the in vitro import assay (Table 4 and Figure 1B). For At4g09000, addition of thermolysin resulted in the detection of a product of smaller size in the in vitro import assay. This indicates that At4g09000 is localized in the outer-envelope, where it is only partially protected against protease treatment.

The At5g42960 protein has been described as the cp outer-envelope protein OEP24.2 (Schleiff et al., 2003) (Table 3). Its location in the cp outer-envelope would be compatible with the lack of a cTP, and is also supported by the fact that OEP24.2 was identified in three independent cp proteomic studies: two on total cp and one on the cp envelope (Table 2).

A cp envelope location for At4g35000 (ascorbate peroxidase APX3) was also supported by both cp envelope proteomic studies considered here. This suggests that At4g35000 is also a true member of the outer-envelope proteome. For APX3, a peroxisomal location has been previously described by studying fluorescent protein fusions (Narendra et al., 2006) and by immunofluorescence and immunoblot analysis (Lisenbee et al., 2003). This, together with the finding that GOX2 is targeted to both peroxisomes and chloroplasts (see above) and the identification of the SMP2 protein, which shows triple targeting to chloroplasts, mitochondria, and peroxisomes (Abu-Abied et al., 2009), points to the possibility that—in addition

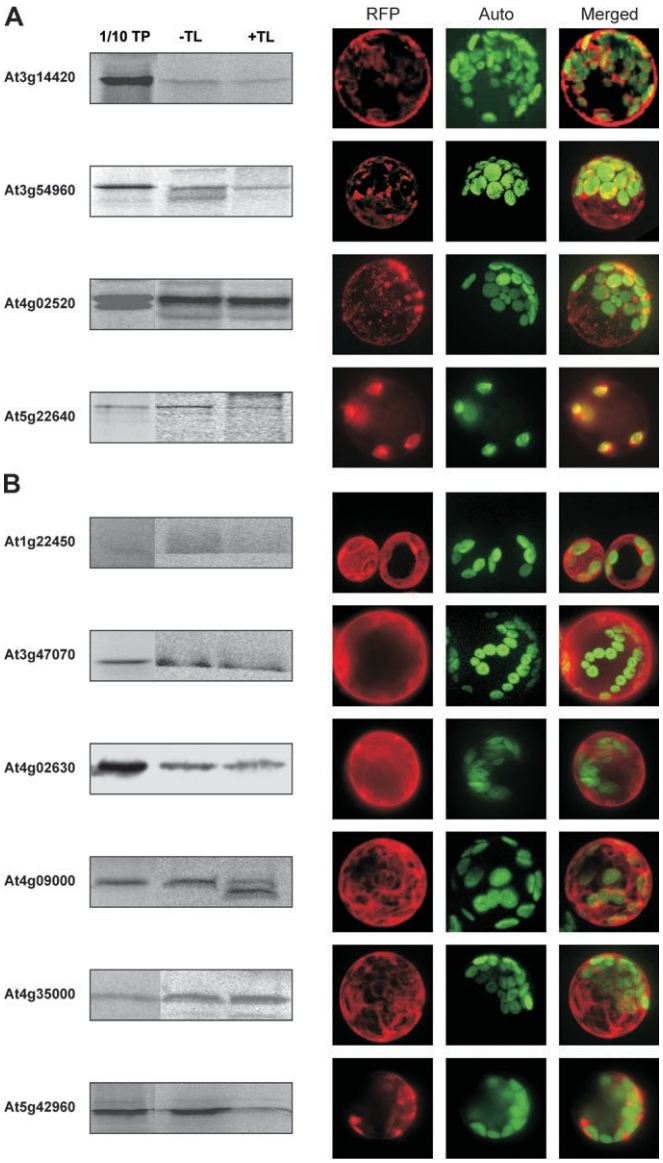

Figure 1. Sub-Cellular Localization of Tentative Non-Canonical CP Proteins by Means of RFP-Fusion and In Vitro Import Assays.

The data shown are for proteins with confirmed (A) or potential (B) cp localization (see text for details). For each protein, in vitro import assays are shown in the left panels. Gels were loaded with 1/10 of the radiolabeled translation product added to the import reaction mixture ('1/10 TP'), and the radiolabeled protein recovered from chloroplasts that had ('+TL') or had not ('–TL') been treated with thermolysin after termination of the import reaction. Results of the corresponding fluorescence micrographs of A. thaliana protoplasts transfected with N-terminal fusions of the entire protein to dsRED are shown in the right panels. The images are presented in false color, with RFP fluorescence ('RFP') in red, chlorophyll autofluorescence ('Auto') in green, and the overlays ('Merged') in yellow.

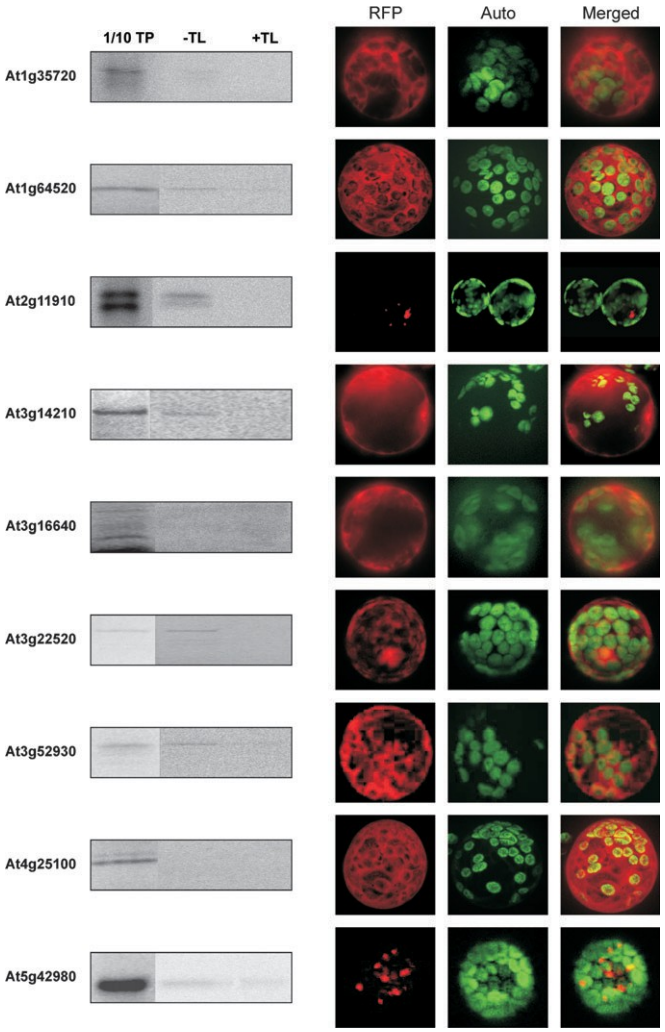

Figure 2. Sub-Cellular Localization by RFP-Fusion and In Vitro Import Assay of Representative Non-Confirmed Tentative Non-Canonical CP Proteins.

For details, see the legend to Figure 1.

to dual targeting to mitochondrion-chloroplast (Carrie et al., 2009) and mitochondrion-peroxisome (Carrie et al., 2008), proteins can also be directed to the chloroplast–peroxisome pair in plant cells.

The At4g02630 (TAK1) protein was found to be imported into chloroplasts (Figure 1B and Table 4), but the RFP-fusion assay gave a negative result. Because TAK1 was previously reported to be localized in thylakoids by immuno-localization (Snyders and Kohorn, 1999), it appears likely that fusion to RFP prevents proper cp targeting of this protein.

Half of the Proteins Tested Do Probably Not Localize to the Chloroplast

Of the 28 proteins in our test set, 14 (Table 4 and Figure 2) showed no evidence of cp localization in either of our assays. Interestingly, eight of these were shown to attach to

Table 4. Experimentally Determined Sub-Cellular Location of Tentative Non-Canonical Chloroplast Proteins.

Protein	In vitro import	RFP assay
cp location		
At3g14420	+	+
At3g54960	+	+
At4g02520	+	+
At5g22640	+	++
Potential cp location		
At1g22450	+	Membranes
At3g47070	+	Cytosol, membranes
At4g02630	+	Cytosol
At4g09000	++ *	Cytosol
At4g35000	+	Cytosol
At5g42960	+	Others
Non-cp location		
At1g01540	–	Cytosol
At1g35720	–	Cytosol
At1g64520	(+)	Cytosol
At2g11910	(+)	Others
At3g02520	(+)	Others
At3g14210	(+)	Cytosol, membranes
At3g16640	–	Others
At3g22520	(+)	Nucleus, others
At3g52930	(+)	Cytosol
At4g01330	–	Cytosol
At4g25100	–	Cytosol
At4g39730	(+)	Others
At5g42980	–	Others
At5g51540	(+)	Cytosol
Unclear		
At1g16790	ntp	Others
At1g78380	ntp	Plasmamembrane
At2g45740	ntp	Others
At4g25210	ntp	Plasmamembrane, others

RFP-fusion assay: + signal in chloroplasts and other compartments; ++ signal only in chloroplasts. In vitro import assay: (+) found attached to chloroplasts; + imported into chloroplasts; ++ imported and processed. ntp, no translation product. * Note that At4g09000 was processed by thermolysin and not by the endogenous cp protease that removes cTPs.

chloroplasts in the in vitro import assay—an observation that might explain why they have been identified in the course of proteomic studies of chloroplasts. This sample includes At1g64520 (putative 26S proteasome regulatory subunit) and At3g52930 (putative fructose bisphosphate aldolase), which have also been reported to be located in the cytosol/nucleus (Fu et al., 2001; Smalle et al., 2002) and mitochondria (Giege et al., 2003), respectively. Moreover, in line with our findings, Annexin 1 (At1g35720) was shown by immunolocalization and ^{3}H-galactose incorporation to function in Golgi-mediated secretion (Clark et al., 2005). Interestingly, TAK2

and TAK3 failed to localize in chloroplasts in both assays and did not even attach to chloroplasts in the in vitro import assay. This indicates that the signals assigned to these proteins in immuno-localization experiments (Snyders and Kohorn, 1999) were probably due to contamination.

Four Members of the Test Set Could Not Be Definitively Localized

The in vitro import assay could not be performed on a further group of four proteins because in vitro expression products could not be obtained. Since RFP-fusion assays did not provide any evidence for a chloroplast destination of these test proteins in the chloroplast, it remains elusive whether any of them resides in the organelle (Table 4). For PEX11 (At2g45740), an extra-chloroplast location is supported by confocal microscopy analyses of fluorescent protein fusion proteins and immunobiochemical analysis, which report the protein to be exclusively located in peroxisomes (Orth et al., 2007).

Functionality of the Predicted SP Sequences of At3g54960 and At3g14210

Four of the tentative non-canonical cp proteins were predicted to contain an SP (see above), including At3g54960 (shown in this study to represent a genuine non-canonical cp protein) and At3g14210 (see Table 4). To test whether these two proteins are actually imported into the ER and processed, in vitro translation was carried out in the presence of dog pancreatic microsomes as in Villarejo et al. (2005). For At3g14210, the translation products detected clearly differed between translation assays with and without microsomes (Supplemental Figure 1A). This implies that At3g14210 contains a functional SP and enters the ER. Because At3g14210 localizes in the cytoplasm and membranes when fused to RFP (Figure 2 and Table 4), its final sub-cellular location is probably not the ER. In combination, these findings suggest that the protein reaches its ultimate sub-cellular destination via the ER. As it is well known that stressed protoplasts tend to show autofluorescence in the 565–620-nm range, the cytoplasmic signal might not be due to the RFP. Hence, the evidence for a cytosolic location for At3g14210 cannot yet be regarded as conclusive.

In the case of the cp protein At3g54960, the size of the translation product did not markedly differ between the assays with and without microsomes (Supplemental Figure 1A). However, the expected size difference due to removal of the SP is only 2.5 kDa and this might easily be masked by glycosylation of the protein. Five N-glycosylation sites are predicted for At3g54960 by the NetNGlyc server (www.cbs.dtu.dk/services/NetNGlyc) (Gupta and Brunak, 2002), which has been programmed for N-glycosylation sites in humans, but has already been successfully employed for plant proteins (Bykova et al., 2006) (Supplemental Figure 1B). The fact that in the in vitro import assays, At3g54960 is imported in an ER-independent fashion argues that passage through the ER is not obligatory for cp targeting of At3g54960. However, the possibility remains that At3g54960 enters the chloroplast via two redundant pathways.

DISCUSSION

Ten of the 28 tentative non-canonical cp proteins examined here, a small subset of those identified in earlier proteomics studies, are (probably) located in chloroplasts; indeed, four of them could be unambiguously shown actually to reside in the organelle (see Table 4). An additional six passed only one of our two tests, and were classified as potential cp proteins. Of this latter sextet, one (At4g09000, 14–3–3-like protein GRF1 chi) obviously is located in the cp outer-envelope and contains a domain that is accessible to external proteases, as indicated by the fact that it is processed by thermolysin in the in vitro import assay. Another (At5g42960, OEP24.2) is a member of the group of cp outer-envelope proteins that are known to lack a cTP (Soll and Schleiff, 2004; Jarvis, 2008). These findings clearly indicate that the fraction of cp proteins that are targeted to inner compartments of the organelle despite lacking a cTP is most likely not as large as previously proposed (Kleffmann et al., 2004). Our data also provide a plausible explanation for the occurrence of false positives observed in cp proteomics studies. Most of the proteins tentatively assigned to chloroplasts on the basis of cp proteomics but not found to enter the organelle in our study clearly attached to the organelle in the in vitro import assay (see Table 4). This indicates that these proteins might associate loosely with the cp outer membrane in a similar fashion as glycolytic enzymes adhere to the mitochondrial outer membrane (Giege et al., 2003), and therefore represent prime sources of contamination in cp preparations, particularly those comprising total chloroplasts or cp envelopes (see Table 1).

These inferences, of course, depend on the assumption that the combination of RFP-fusion and in vitro import assays provides a reliable experimental validation of cp location. Only proteins that could be localized to the organelle in both assays are considered to be true cp proteins, although it cannot be absolutely excluded that cp proteins exist for which both assays fail. Those proteins that were tested negative in the RFP-fusion assay but were imported into chloroplasts in vitro are nevertheless regarded as potential cp proteins, based on the observation that fusion of RFP to the C-terminus of bona fide cp proteins often prevents their import into chloroplasts. Examples include the cp proteins TAK1 and TSP9 that have been described to be associated with thylakoids based on immunolocalization studies (Snyders and Kohorn, 1999; Carlberg et al., 2003), but failed to yield cp signals in RFP-fusion assays (this study), as well as outer-envelope b-barrel proteins (Katrin Philippar, personal communication). It therefore seems likely that most, if not all, potential cp proteins identified in our study are indeed located in chloroplasts, despite the negative RFP-fusion assay results. In this context, At4g35000 might represent a true envelope-located ascorbate peroxidase. Moreover, it is surprising that At5g51540 (metalloendopeptidase),

At1g16790 (ribosomal protein-related), and At3g22520 (unknown protein) were not positively tested in our two assays, although they have been repeatedly and exclusively identified in cp proteomic studies (see Tables 2 and 4). If they truly represent cp proteins (as suggested by the proteomic studies), their failure to behave as expected in in vitro import assays implies that additional factors might be required for their uptake in vitro and that, in addition to in vitro import and RFP-fusion assays, also immunoblot analyses are required to unequivocally exclude a cp location for such proteins. How large is the fraction of non-canoncial cp proteins in A. thaliana? Based on our analysis of the test set of 28 tentative cp proteins without cTP, it can be extrapolated that as many as 343 proteins (or 11.4%) might contribute to the fraction of non-canonical cp proteins in Arabidopsis chloroplasts (Table 5).

The initial suggestion that the chloroplast proteome consists of a large fraction of proteins lacking a cTP (Kleffmann et al., 2004) can be interpreted in two ways. First, these proteins might not possess any cleavable targeting pre-sequence (i.e. non-canonical cp proteins) or, second, they carry these presequences, but available prediction algorithms fail to recognize them. Our data, from a combination of two chloroplast localization methods, indicate (1) that the fraction of non-cTP cp proteins is actually not as large as proposed, and (2) that none of them possesses a cleavable presequence. This strongly argues that insufficient sensitivity of cTP predictions is not a significant issue—at least when the available methods are used in combination, as in our study. The specificity of cTP prediction, namely how many of the predicted cTPs truly belong to cp proteins, is more difficult to assess, because the complete repertoire of cp proteins has not yet been defined, making it impossible to unambiguously identify false positive cTP predictions. A further factor that complicates cTP prediction is the fact that a certain ambiguity between cp and mitochondrial targeting exists (Chew and Whelan, 2004). Thus, some proteins have pre-sequences that target them to both organelles (Silva-Filho, 2003; Duchene et al., 2005; Pesaresi et al., 2006), and instances of cTPs predicted as mTPs are also known, such as CRR6 (Munshi et al., 2006) and LHCA5 (Ganeteg et al., 2004; Lucinski et al., 2006). It appears likely that many more genuine cp proteins exist that contain presequences that resemble mTPs; possible candidates include tentative cp proteins repeatedly identified in cp proteomics studies and containing a predicted mTP, such as At4g25450 (Ferro et al., 2003; Froehlich et al., 2003; Zybailov et al., 2008) and At2g31670 (Kleffmann et al., 2004; Peltier et al., 2006; Rutschow et al., 2008; Zybailov et al., 2008).

Table 5. Extrapolation of the Abundance of Non-Canonical cp Proteins.

	This study [1]	Extrapolation to all 445 tentative non-canonical cp proteins[2]	Extrapolation to all 3000 cp proteins[3]
cp location	4 (14.3%)	64	137 (4.6%)
Potential cp location	6 (21.4%)	95	206 (6.8%)
Total	10 (35.7%)	159	343 (11.4%)

1 Absolute numbers are derived from Table 4, and relative numbers refer to the total number of 28 proteins tested in our study.
2 For this extrapolation, the fraction size (in %) in column 2 was multiplied by 445, the number of cp proteins without cTP identified in Table 1.
3 This number was obtained by multiplying the numbers provided in column 3 with the factor 3000/1392, with 1392 being the total number of tentative cp proteins listed in Table 1 and 3000 the estimated total number of cp proteins according to Abdallah et al. (2000) and Leister (2003).

Future analyses will clarify whether and to what extent the novel non-cTP proteins identified in this study are targeted to the chloroplast by a Toc/Tic-independent translocation mechanism. In addition, it will be of interest to study localization of our test set proteins with further methods, in order to refine our data by extrapolating if and how many cp proteins do exist that fail detection by either in vitro import or RFP-fusion assay. Moreover, more exotic contributions to the cp proteome, such as the import of cytosolic mRNA into chloroplasts, as described for the mRNA encoding the eukaryotic translation factor 4E (Nicolai et al., 2007), must also be considered in forthcoming systematic analyses of the cp proteome.

METHODS

Prediction of Sub-Cellular Location

Amino-acid sequences of proteins were obtained from the TAIR database (www.arabidopsis.org/index.jsp) and analyzed with the following prediction programs (all are available online): TargetP (Emanuelsson et al., 2007); ChloroP (Emanuelsson et al., 2000); Predotar (Small et al., 2004); iPSORT (Bannai et al., 2002); WoLF PSORT (Horton et al., 2007); PSORT (Nakai and Horton, 2007); BaCelLo (Pierleoni et al., 2006); PCLR (Schein et al., 2001); ProteinProwler (Hawkins and Boden, 2006).

In Vitro Transcription and Translation

Full-length cDNAs were cloned downstream of the T7 or SP6 promoter in the vector pGEM-T (Promega). mRNAs were then obtained by transcription with T7 or SP6 RNA polymerase (MBI Fermentas, Leon-Rot, Germany) and used for translation in wheat germ (Wheat Germ Extract System, Promega, Madison, WI, USA) or reticulocyte extracts (Flexi, Promega) in the presence of [^{35}S]methionine. All translation mixtures were centrifuged at 50 000 g (1 h; 4°C) prior to import experiments.

Expression in Reticulocyte Lysate in the Presence of Dog Pancreas Microsomes

Translation of At3g54960 and At3g14210 RNA was performed in reticulocyte lysate in the presence or absence of dog pancreas microsomes as described by Villarejo et al. (2005).

Chloroplast Isolation and Protein Import

Chloroplasts were isolated from leaves of 9–11-day-old pea seedlings (P. sativum var. Arvica) and purified by centrifugation through Percoll density gradients as described (Vojta et al., 2007). A standard import reaction contained chloroplasts equivalent to 15–20 μg chlorophyll in 100 μl of import buffer (330 mM sorbitol, 50 mM HEPES/KOH pH 7.6, 3 mM $MgSO_4$, 10 mM Met, 10 mM Cys, 20 mM potassium gluconate, 10 mM $NaHCO_3$, 2% (w/v) BSA), and up to 5% (v/v) of an ^{35}S-labeled translation reaction. Import reactions were initiated by the addition of translation product and carried out for 20 min at 25°C. Reactions were terminated by collecting chloroplasts from the reaction mixture by centrifugation through a 40% (v/v) Percoll cushion. Chloroplast were washed once, solubilized and fractionated by SDS–PAGE. Radiolabeled proteins were then analyzed with a phosphor-imager.

In order to distinguish envelope-associated from imported proteins, chloroplasts were treated with the protease thermolysin (20 μg ml^{-1}) for 20 min on ice (Waegemann and Soll, 1995) after import. In such cases, chloroplasts were purified through Percoll density gradients prior to further use (Waegemann and Soll, 1995). To investigate whether import signals after thermolysin treatment are truly due to localization of the proteins inside the chloroplasts and not to protection by other proteins, after successful import reaction chloroplasts were first lysed by addition of 1% b-dodecyl-maltoside and then treated with thermolysin.

Intracellular Localization of dsRED Fusions

The Red Fluorescent Protein from the reef coral Discosoma (dsRED) (Jach et al., 2001) was used as a reporter to determine the intracellular localization of the proteins in transient gene expression assays. The coding regions of the genes analyzed were amplified and cloned upstream of the dsRed sequence using the NcoI restriction enzyme site. Sterile cotyledons of 2-week-old plants (ecotype ColGl-1) were cut into small pieces and incubated for 16 h at 24°C in the dark in a protoplasting solution (10 mM MES, 20 mM $CaCl_2$, 0.5 M mannitol pH 5.8, 0.1 g ml^{-1} macerozyme (Duchefa), 0.1 g ml^{-1} cellulase (Duchefa)) followed by the isolation of protoplasts as described in Dovzhenko et al. (2003). Plasmid DNA (40 μg) was introduced into protoplasts by PEG-mediated transfection as previously described (Koop et al., 1996). Preparations were viewed with a Fluorescence Axio Imager microscope in ApoTome mode (Zeiss) after 16 h of incubation at 23°C in the dark. Fluorescence was excited with the X-Cite Series 120 fluorescence lamp (EXFO) and images were collected in the 565–620-nm (dsRED fluorescence) and 670–750-nm (chlorophyll autofluorescence) ranges.

SUPPLEMENTARY DATA

Supplementary Data are available at Molecular Plant Online.

FUNDING

This work was supported by the Deutsche Forschungsgemeinschaft (Grant SFB-TR1 TP A10 to D.L.).

ACKNOWLEDGMENTS

We thank Paul Hardy for critical comments on the manuscript, and Gisela Arnold, Christine Hümmer, and Alma Tursic for excellent technical support. No conflict of interest declared.

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