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The role of the N-terminal domain of chloroplast targeting peptides in organellar protein import and miss-sorting

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Abstract **We have analysed 385 mitochondrial and 567 chloroplastic signal sequences of proteins found in the organellar proteomes of *Arabidopsis thaliana*. Despite overall similarities, the first 16 residues of transit peptides differ remarkably. To test the hypothesis that the N-terminally truncated transit peptides would redirect chloroplastic precursor proteins to mitochondria, we studied import of the N-terminal deletion mutants of ELIP, PetC and Lhcb2.1. The results show that the deletion mutants were neither imported into chloroplasts nor miss-targeted to mitochondria *in vitro* and *in vivo*, showing that the entire transit peptide is necessary for correct targeting as well as miss-sorting.**
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Keywords: Chloroplast; Mitochondria; Targeting peptide; Protein import; Miss-sorting

1. Introduction

More than 90% of mitochondrial and chloroplastic proteins are encoded in the nucleus and synthesized in the cytosol as precursors carrying a cleavable N-terminal signal- or targeting-peptide. The signal peptide, also called presequence in mitochondria and transit peptide in chloroplasts, carries the information required for targeting to the correct organelle. The sorting mechanisms are not fully understood, but it is clear that organelle specific receptors recognise the signal peptides and guide the protein to the import pores of the translocase complexes located in the outer and inner mitochondrial membranes (TOM and TIM) and chloroplastic envelopes (TOC and TIC). After completed import, the targeting peptide is cleaved off by the mitochondrial or the stromal processing peptidase (for reviews see [1–3]).

Apart from the fact that chloroplast transit peptides are on average longer than mitochondrial presequences (58 residues vs. 42 residues), they are remarkably similar with respect to the amino acids composition. They exhibit high abundance of hydroxylated, hydrophobic and positively charged amino acids and very low abundance of acidic amino acid residues [4]. Notably, plant mitochondrial presequences, in contrast to presequences from non-plant sources, but in accordance with transit peptides, are rich in serine. Some structural differences have been reported. Mitochondrial presequences form amphipathic α-helices [5,6], which are important for import [7], whereas chloroplast transit peptides are generally unstructured. However, transit peptides also form helices upon membrane contact [8]. This high similarity of presequences and transit peptides is puzzling as it leaves a decisive question of protein sorting unanswered: How does the cell distinguish between mitochondrial and chloroplast targeting peptides? Which features determine the destiny of preproteins? This question is crucial for life as miss-sorting would have disastrous effects on organellar functions.

Studies with dually targeted proteins, i.e. proteins encoded by a single gene and targeted to mitochondria as well as chloroplasts, showed a domain organisation of the signal peptide necessary for efficient targeting to mitochondria and chloroplasts [9–11]. A domain structure has also been identified for targeting peptides of proteins being directed to a single organelle, such as chloroplast ferredoxin and the small subunit of Rubisco, where deletions influence different steps of the import process [12,13]. A kind of domain structure can also be seen for a dataset of 58 mitochondrial and 277 chloroplast proteins from different plant sources when analysing the positional abundance of amino acids in SequenceLogos. Arginine and leucine are significantly underrepresented in the N-terminal portion of chloroplast signal peptides, whereas they are very dominant in the N-terminal 'domain' of mitochondrial signal peptides [4]. The distribution of these amino acids in the residual part of the transit peptides, however, resembled the overall amino acid distribution in mitochondrial presequences. This led to an interesting question, if these differences also occur in a single plant species and if N-terminally deleted chloroplast precursors would be targeted to mitochondria.

Here we present new SequenceLogos of all the mitochondrial [14] and chloroplastic [15] proteins found in organellar proteomes from a single plant species, *Arabidopsis thaliana*. Based on the results, we have investigated import of the N-terminal deletion mutants for ELIP, PetC and Lhcb2.1 precursors to test the hypothesis of redirection of these proteins to mitochondria. Unexpectedly, the full-length PetC and Lhcb2.1 precursors were *in vitro* miss-targeted to mitochondria. Furthermore, the *in vitro* and *in vivo* import of the deletion mutants was completely abolished into both organelles.

2. Materials and methods

2.1. Sequence analysis (SequenceLogos)

For sequence analysis and logo creation, sequences longer than 200 amino acids were selected from *A. thaliana*, 385 proteins of the mitochondrial proteome [14] and 567 proteins of the chloroplast

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proteome [15]. SequenceLogos were created using seqlogo in the version 2.8.1 [16,17] from the first 60 amino acids of the selected proteins. Amino acid contents were calculated for the whole proteomes, the amino acids 2–40 (mitochondria) or 2–60 (chloroplasts) and for amino acids 2–16. For statistical evaluation, 1000 random datasets were created for each proteome. Each random dataset had the same size and amino acid composition as the corresponding organellar proteome.

Amino acid differences were calculated as difference of the content in chloroplasts and the content in mitochondria and represented in percent of the mitochondrial content. Amino acid content differences were considered significant, if the difference was larger than four standard deviations of the differences in the random datasets.

2.2. TargetP and Predotar prediction programs

TargetP version 1.1 [18] was used in the plant mode without cut-offs, however, including cleavage site prediction (http://www.cbs.dtu.dk/services/TargetP/). Predotar [19] was used with standard settings (http://www.inra.fr/predotar/).

2.3. Generation of in vitro constructs

All cDNAs were PCR amplified from different sources. ELIP was amplified using the 5′-(ATGGCAACAGCATCGTTC) and 3′-primers (TTAGACGAGTGTCCCACCTTTG) from a λPR2 vector. PetC and Lhcb2.1 were amplified from linear DNA fragments (provided by Prof. I. Adamska) using primer pair (5′: ACAATGGCG TCCTCATCC; 3′: TTAAGACCACCATGGAGCATC) and (5′: ACGATAATGGCAACATCAGCT; 3′: TTAATTTCCGGGGACAAA GTT), respectively. Deletion mutants were constructed using the following primer pairs: (Δ2–15)ELIP, 5′: *AGATCT*ATGTTAACCACTCGCAAGATCAAC, 3′: *GGTACC*ACCGCTAAACGCTAG CAAG; (Δ2–13)PetC, 5′: *AGATCT*ATGTCTA GCAGAAGTGCTTT GATG, 3′: *GGTACC* ACCTCCGGTTCCAGGAGG and (Δ2–12) Lhcb2.1, 5′ *AGATCT*ATGGGCCAAACGGCT CTCAAG, 3′: *GGTACC*GTCCCAGCCGTAGTCTCC. The PCR products were cloned into pCR®-Blunt II-TOPO® using the Zero Blunt® TOPO® PCR cloning kit (Invitrogen) according to the manufacturer's specifications.

2.4. In vitro import into mitochondria and chloroplasts

All the translated products were synthesized using the *in vitro* transcription/translated coupled reticulocyte lysate TNT system (SDS-Promega) in the presence of [^{35}S]-methionine (Amersham).

Spinach mitochondria and chloroplasts were isolated from spinach leaves and single and dual import experiments were performed as described by Eriksson *et al.* [20], Bruce *et al.* [21] and Rudhe *et al.* [22].

3. GFP fusion constructs

For the construction of GFP fusion proteins (ΔELIP-GFP, ΔPetC-GFP and ΔLhcb2.1-GFP) the target sequences were amplified from the full-length TOPO *in vitro* constructs using the same 5′Δ primers as above. The 3′primers were designed to allow the amplification of the targeting sequence plus 50 residues of the mature portion of the protein. The PCR products were cloned into pCR®-Blunt II-TOPO® and transferred to the pTZ19U vector [23] between the pma4-35S promoter and GFP. In order to perform *Agrobacterium tumefaciens* mediated transient expression, the constructs were inserted into a pBI101 vector (Clontech, [24]).

3.1. Transformation into Agrobacterium and transfection of tobacco leaves

A. tumefaciens C58 cells were transformed with the pBI101 constructs. *Agrobacterium*-mediated transient expression into tobacco leaves was carried out as described by Batoko *et al.* [25].

3.2. Confocal microscopy analysis of in vivo constructs

Confocal microscopy was performed by the Carl Zeiss Laser Scanning System LSM 510 and the Zeiss LSM Image Browser

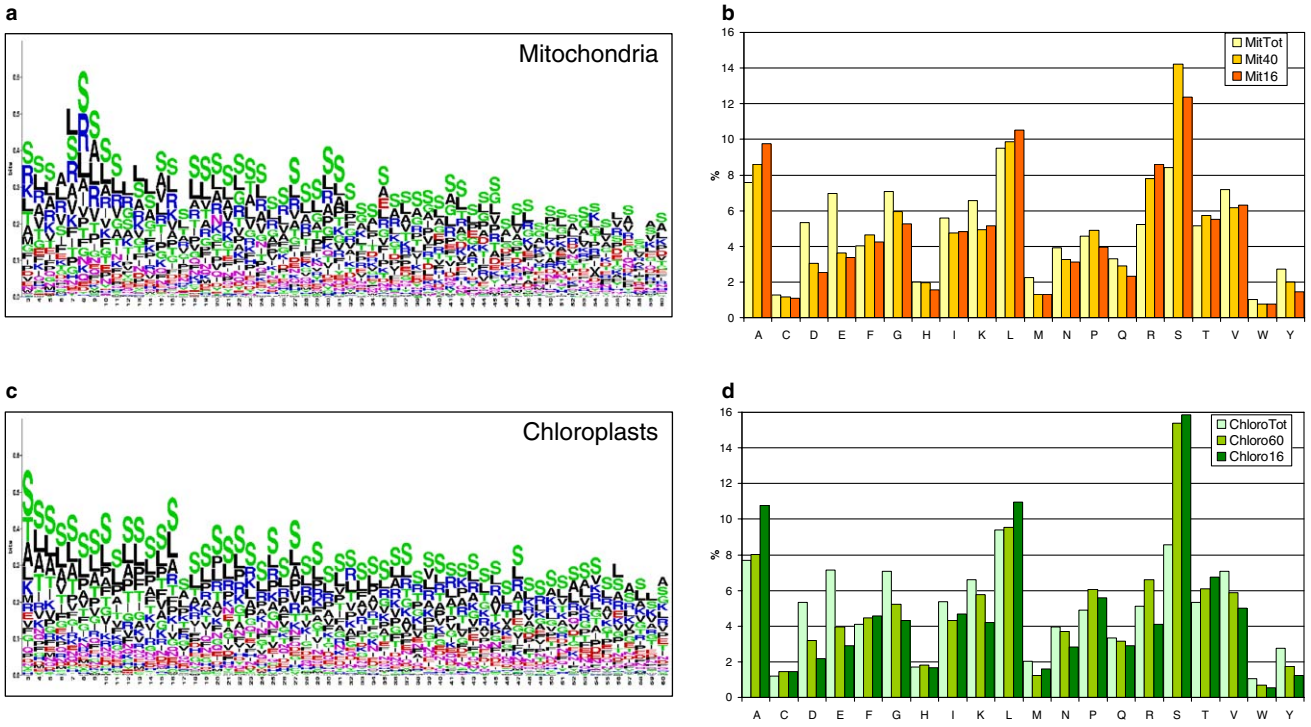

Fig. 1. Analysis of the amino acid composition of the N-terminal part of proteins from the mitochondrial and chloroplastic proteomes. SequenceLogos for 385 mitochondrial (a) and 567 chloroplastic proteins (c) show a clear difference in arginine content in the first 16 amino acids. The global amino acid composition is marginally different between the two proteomes and the whole targeting peptide, but shows a major difference in arginine content in the first 16 amino acids (b,d).

Table 1
Amino acid composition in chloroplastic and mitochondrial targeting sequences

	Mit 2–40	Difference1 (%)	Chloro 2–60	Mit 2–16	Difference2 (%)	Chloro 2–16
A	8.61	−7	8.03	9.75	10	10.77
C	1.17	**23**	1.44	1.09	32	1.44
D	3.03	5	3.19	2.55	−14	2.19
E	3.63	**9**	3.94	3.36	−13	2.91
F	4.64	−4	4.47	4.26	7	4.56
G	5.96	**−12**	5.24	5.27	−18	4.33
H	1.96	−7	1.83	1.57	6	1.67
I	4.74	−9	4.33	4.82	−3	4.67
K	4.93	**17**	5.78	5.17	−19	4.21
L	9.87	−3	9.56	10.51	4	10.96
M	1.31	−7	1.22	1.31	21	1.59
N	3.27	13	3.70	3.11	−9	2.83
P	4.90	**24**	6.06	3.97	**41**	5.60
Q	2.89	9	3.16	2.34	24	2.91
R	7.79	**−15**	6.60	8.61	**−52**	4.09
S	14.21	**8**	15.4	12.38	**28**	15.86
T	5.72	6	6.08	5.52	22	6.74
V	6.16	−4	5.89	6.30	**−21**	4.99
W	0.78	−12	0.69	0.76	−26	0.56
Y	2.00	−13	1.75	1.46	−15	1.24

Difference 1: difference between the amino acid content in amino acids 2–40 for mitochondria and the amino acids 2–60 for chloroplasts (average length targeting peptide) in % of the mitochondrial content. Difference 2: difference between the amino acid content in amino acids 2–16 for mitochondria and chloroplasts in % of the mitochondrial content. Differences in bold are significant.

Version 3,1,0,99. For the GFP detection, the excitation was at 488 nm and the detection between 506 and 538 nm. Chloroplast autofluorescence was detected between 664 and 696 nm with an excitation at 488 nm.

4. Results and discussion

4.1. Sequence analysis

In order to get detailed information on the amino acid composition and distribution in mitochondrial presequences and chloroplastic transit peptides from a single plant source, we analysed all proteins found in the mitochondrial and chloroplastic proteomes of *A. thaliana*. The mitochondrial proteome includes 385 proteins [14] and the chloroplastic, 567 proteins [15]. We calculated overall sequence composition for an average targeting peptide consisting of 40 and 60 amino acids for mitochondrial and chloroplastic targeting peptides, respectively. At the selected cut-offs, the amount of acidic amino acid residues increases significantly, indicating the start of the ''mature'' protein. The overall composition of both the mitochondrial and chloroplastic targeting peptides is 33–35% hydrophobic, 22–23% hydroxylated and 14–15% positively charged amino acids. Furthermore, proline and glycine constitute about 11%. The differences between the two organelles are very small (Fig. 1b

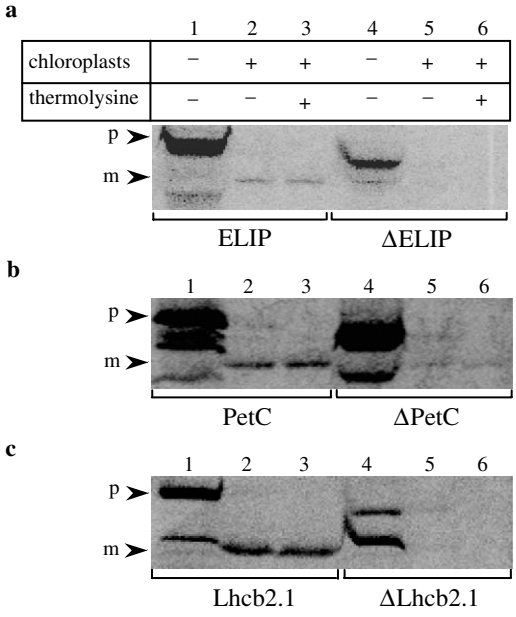

Fig. 2. In vitro import into chloroplasts. ELIP and ΔELIP (a), PetC and ΔPetC (b) and Lhcb2.1 and ΔLhcb2.1 (c) were incubated with isolated spinach chloroplasts as described in Section 2. Thermolysin (5 μg ml^{-1}) was added after import where indicated.

Table 2
Organellar prediction results from Predotar and TargetP for ELIP, PetC and Lhcb2.1 and their deletion mutants

Program	Predotar		TargetP	
Localisation	Chloroplastic	Mitochondrial	Chloroplastic	Mitochondrial
ELIP	**0.891**	0.020	**0.684**	0.103
Delta(2–15)ELIP	0.016	**0.945**	0.230	**0.374**
PetC	**0.709**	0.001	**0.652**	0.163
Delta(2–13)PetC	0.000	**0.623**	**0.654**	0.185
Lhcb2.1	**0.990**	0.012	**0.919**	0.088
Delta(2–12)Lhcb2.1	0.000	**0.996**	0.102	**0.474**

and d). The significant differences in arginine and proline content are compensated by lysine and glycine, respectively. Serine and cysteine, however, show significant relative increases of 8% and 23% in chloroplast transit peptides (Table 1).

The positional distribution of amino acids is best visualized with SequenceLogos (Figs. 1a and c). We omitted amino acids one (100% methionine) and two (>20% alanine) to avoid very small letters in the important part of the graphs. These two positions showed a very similar amino acid distribution in both datasets. The SequenceLogos show high differences, when analysing the first 16 amino acids of the proteins. The main difference is for arginine that is greatly overrepresented (+52%) in the N-terminal portion of the mitochondrial presequences. This excess of positive charges is not compensated in chloroplast targeting peptides by lysine (+19% in mitochondria). The N-terminal portion of chloroplast targeting peptides on the other hand has a significant excess of serine (+28%) and proline (+41%). In contrast to the results obtained in an earlier study [4] with targeting peptides from different plant sources, we do not find a significantly increased leucine content in *A. thaliana* mitochondrial presequences.

The above-mentioned results and the observation that amino acids 17–60 of chloroplast transit peptides resemble a mitochondrial presequence, led to a hypothesis that N-terminally deleted chloroplast transit peptides would direct proteins into mitochondria.

4.2. Intracellular protein localization prediction (TargetP and Predotar)

To test this hypothesis, we selected three nuclear-encoded chloroplast proteins from *A. thaliana*: ELIP, an early light induced protein involved in light stress response, PetC, the Rieske iron–sulfur protein of the cytochrome b_6f complex in the thylakoid membrane and Lhcb2.1, a light harvesting protein of the PSII antennae. The selected proteins fit well the prediction, ELIP and Lhcb2.1 do not contain any arginine in the N-terminal portion of the targeting peptides, whereas PetC contains one arginine. All chloroplastic sequences contain at least 3 serines or threonines, whereas the targeting signal of the mitochondrial $F_1\beta$ contains 3 arginines and 3 serines. For each of the chloroplast precursor proteins we created a mutant with an N-terminal deletion. We predicted the intracellular localisation for all of these proteins using TargetP and Predotar. When applying the wild-type sequences of the proteins, both TargetP and Predotar predict the correct localisation for the selected proteins (Table 2). However, when deleting 12–15 N-terminal residues of the chloroplast presequences, Predotar predicts a miss-sorting of these proteins to

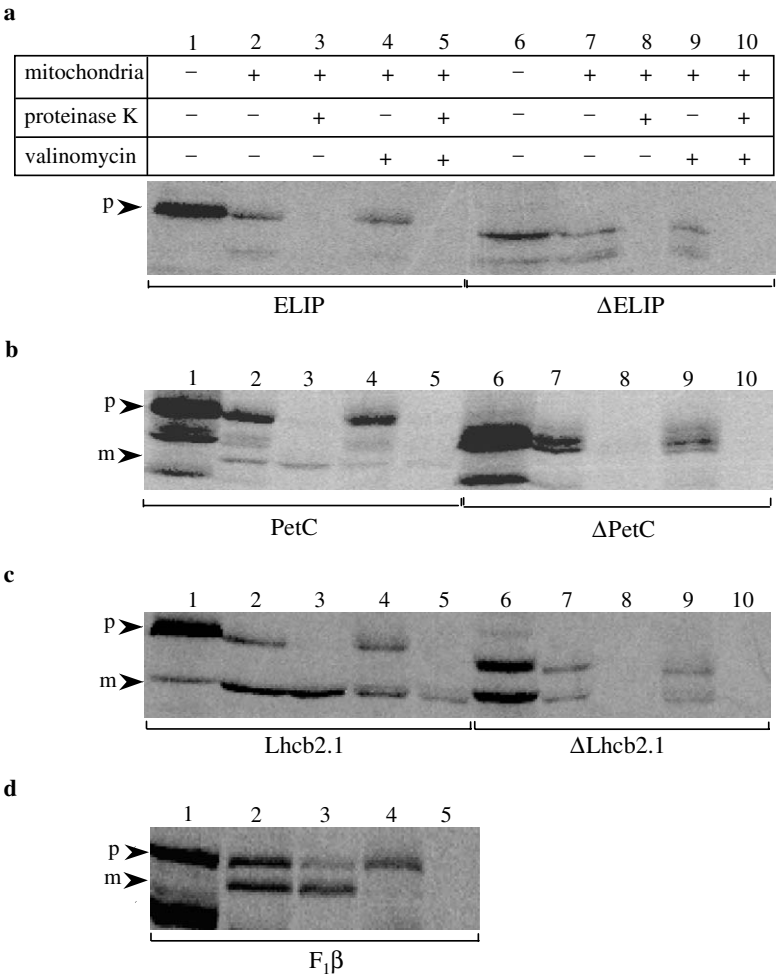

Fig. 3. In vitro import into mitochondria. ELIP and ΔELIP (a), PetC and ΔPetC (b), Lhcb2.1 and ΔLhcb2.1 (c) and $F_1\beta$ (d) were incubated with isolated spinach mitochondria as described in Section 2. Proteinase K (15 μg ml^{-1}) was added after import. 1 μM valinomycin was added before import where indicated.

mitochondria with high probability (0.62–0.99). TargetP on the other hand shows a mixed result. It does not change its prediction for the PetC deletion mutant, Δ(2–13)PetC is still predicted to be a chloroplastic protein, but Δ(2–15)ELIP and Δ(2–12)Lhcb2.1 are predicted to be mitochondrial proteins. However, the prediction by TargetP is of low probability with the reliability class of 5 [18].

4.3. In vitro import into chloroplasts

Chloroplastic targeting of the ELIP, PetC and Lhcb2.1 precursors and their deletion mutants was investigated using *in vitro* import into isolated spinach chloroplasts (Fig. 2). Incubation of the ELIP, PetC and Lhcb2.1 precursors with isolated chloroplasts resulted in import and processing of the precursor proteins. After thermolysin treatment of the chloroplasts only the mature forms were found to be protected inside the organelle. Incubation of the ΔELIP and ΔLhcb2.1 precursors with isolated chloroplasts resulted in neither import nor processing of these precursors into chloroplasts and the import of ΔPetC was reduced by more than 80%. These results show that the full length precursors of ELIP, PetC and Lhcb2.1 are imported and processed into chloroplasts, and that the extreme N-terminal part of the targeting signal is necessary for efficient translocation, which is in agreement with previously published results for ferredoxin [12] and SSU [26].

4.4. In vitro import into mitochondria

Miss-sorting and redirection of ELIP, PetC and Lhcb2.1 and their deletion mutants was studied *in vitro* with isolated spinach mitochondria (Fig. 3). Incubation of ELIP precursor with isolated mitochondria resulted in neither import nor processing, but interestingly, PetC and Lhcb2.1 precursors were imported and processed inside mitochondria. After PK treatment of the mitochondria, the mature forms of these precursors were protected inside mitochondria. The addition of valinomycin did not completely abolish import of PetC and Lhcb2.1 indicating partial membrane potential independent import of these precursors into the mitochondria. This phenomenon has also been seen for authentic mitochondrial precursors [22]. *In vitro* miss-targeting of chloroplast precursors into mitochondria has been observed earlier for a range of proteins (for review see [1]). The mechanism of miss-sorting is not fully understood, but the miss-sorting has been shown to be transit peptide dependent [27]. The presence of chloroplasts during import reduced the miss-sorting [11]. The requirement of cytosolic factors for correct sorting has been suggested as miss-sorting does not occur *in vivo*.

In contrast to the full-length precursors, incubation of the N-terminally truncated mutants, ΔELIP, ΔPetC and ΔLhcb2.1 with isolated mitochondria resulted in neither import nor processing of these precursors in mitochondria (Fig. 3). This is in contrast to our hypothesis and *in silico* prediction and shows that removing 12–15 N-terminal amino acids from a chloroplast transit peptide, does not redirect the proteins from chloroplasts to mitochondria, but inhibits import into both organelles. The N-terminal portion of the transit peptides is not only required for choroplastic import but is also involved in the miss-targeting to mitochondria.

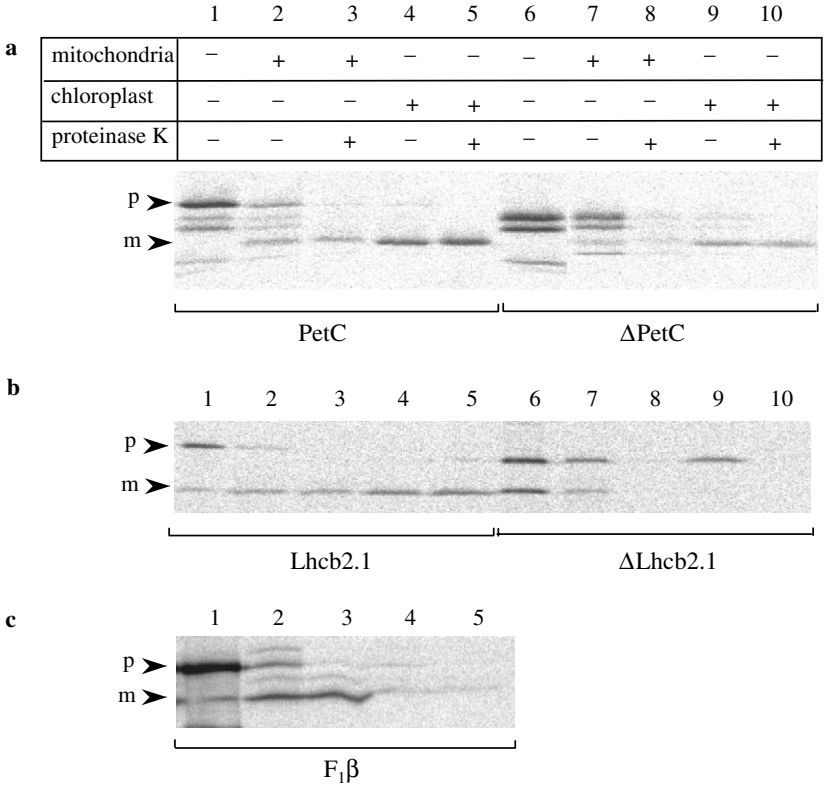

Fig. 4. Dual in vitro import in mitochondria and chloroplasts. PetC and ΔPetC (a) Lhcb2.1 and ΔLhcb2.1 (b) and $F_1\beta$ (c) were incubated with isolated mitochondria and chloroplasts in the same reaction mixture. Mitochondria and chloroplasts were reisolated on a 15% Percoll gradient after import as described in [22]. Proteinase K (10 μg μl^{-1}) was added after reisolation of the mitochondria and chloroplasts as indicated. p, precursor; m, mature.

4.5. Dual in vitro import into mitochondria and chloroplasts

Import of the PetC, ΔPetC, Lhcb2.1 and ΔLhcb2.1 precursors (Fig. 4a–b) was also tested using the dual *in vitro* import system in which the precursor is simultaneously incubated with both isolated mitochondria and chloroplasts. It has previously been shown that miss-targeting of the small subunit of Rubisco (SSU) to mitochondria is avoided in this system [22]. Both full length precursors (PetC and Lhcb2.1) were imported and processed to a mature size protein in mitochondria and chloroplasts to the same extent as in the single in vitro import experiments. ΔPetC was only imported into chloroplasts. ΔLhcb2.1 was not imported into either organelle, as in the single in vitro import experiments above. A known mitochondrial precursor of the ATP synthase $F_1\beta$-subunit from *Nicotiana plumbaginifolia* was used as a control for import (Fig. 4c). These results show that the *in vitro* miss-targeting of the Lhcb2.1 and PetC precursors to mitochondria cannot be eliminated by the presence of chloroplasts. Import of ΔPetC is reduced in chloroplasts compared to the full-length PetC precursor. The rate of reduction is somewhat different for the single and dual import system but always in the range between 70% and 90% as shown in Figs. 2 and 4.

4.6. In vivo import into tobacco leaves

To analyse *in vivo* targeting or redirection of the deletion mutants of ELIP, Lhcb2.1 and PetC, the targeting peptide and 48 (PetC) or 50 (ELIP and Lhcb2.1) amino acid residues of the mature part of the protein (to preserve the processing site) were fused to GFP, under the plant strong transcription promoter EN50PMA4 [28]. A known mitochondrial targeting peptide of the $F_1\beta$ subunit of the ATP synthase from *N. plumbaginifolia* and a known dual targeted peptide of the presequence protease PreP1 from *A. thaliana* fused to GFP ($F_1\beta$-GFP and *At*PreP1-GFP), were used as controls for mitochondrial targeting [23] and dual targeting [29] to mitochondria and chloroplasts, respectively. *Agrobacterium*-mediated transient expression of the controls and ΔELIP-GFP, ΔPetC-GFP and ΔLhcb2.1-GFP fusion proteins was performed in tobacco leaves and targeting was analysed by confocal microscopy.

When tobacco leaves were infiltrated with the *Agrobacterium* suspension carrying the control $F_1\beta$-GFP construct, green fluorescence was detected in the characteristic mitochondrial structures of small punctuated morphology (Fig. 5a–c) indicating mitochondrial targeting. With the PreP-GFP construct, GFP was dually targeted to the punctuated shape structures, i.e. mitochondria, and large round shape structures (Fig. 5d–f). GFP fluorescence in the large round shape structures co-localized with the chloroplasts as shown by the superimposing of GFP and chlorophyll autofluorescence (Fig. 5f).

The results of the infiltration for ΔELIP-GFP, ΔPetC-GFP and ΔLhcb2.1-GFP constructs showed GFP fluorescence only in the cytosol and the nucleus (Fig. 5g–o), showing that the truncated proteins were neither imported into chloroplasts nor miss-targeted to mitochondria.

Altogether our results present amino acid distribution in mitochondrial and chloroplast targeting peptides derived from several hundred proteins found in the organellar proteomes of *A. thaliana* and show that the N-terminal portion of transit peptides that remarkably differs from the rest of the targeting peptides is not only required for the chloroplastic targeting but is also involved in the *in vitro* miss-targeting of chloroplast precursors to mitochondria.

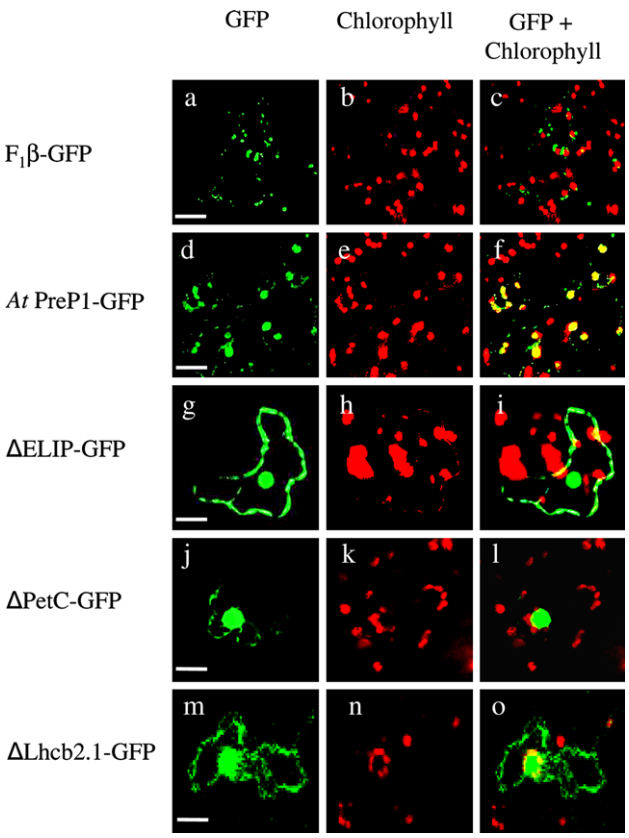

Fig. 5. In vivo targeting of the GFP-fusions to tobacco-leaf epidermal cells. *Agrobacterium*-mediated transient expression of the green fluorescent protein (GFP) fusion constructs into the *N. tabaccum* leaves: F1β-GFP (a–c), AtPreP1-GFP (d–f) ΔELIP-GFP (g–i), ΔPetC-GFP (j–l), and [ΔLhcb2.1-GFP (m–o) as described in Section 2. The GFP column shows the signal detected in the green channel; the chlorophyll column shows the signal detected in the far-red channel and the GFP + chlorophyll column corresponds to the merging of the two previous columns, in which yellow represents the superposition of green and red. Scale bars, 10 μm.

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