Arabidopsis thaliana ferrochelatase-I and -II are not imported into *Arabidopsis* mitochondria

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Abstract Using in vitro import assays into purified mitochondria and chloroplasts we found that *Arabidopsis* ferrochelatase-I and ferrochelatase-II were not imported into mitochondria purified from *Arabidopsis* (or several other plants) but were imported into pea leaf chloroplasts. Other dual targeted proteins could be imported into purified mitochondria from *Arabidopsis*. As only two ferrochelatase genes are present in the completed *Arabidopsis* genome, the presence of ferrochelatase activity in plant mitochondria needs to be re-evaluated. Previous reports of *Arabidopsis* ferrochelatase-I import into pea mitochondria are due to the fact that pea leaf (and root) mitochondria appear to import a variety, but not all chloroplast proteins. Thus pea mitochondria are not a suitable system to either study dual targeting, or to distinguish between isozymes present in mitochondria and chloroplasts. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Mitochondrion; Chloroplast; Protein import; Dual targeting; Mis-targeting

1. Introduction

The specificity of targeting to mitochondria and chloroplasts in plant cells has been a much studied topic since the initial report by Hurt et al. in 1986 that the *Chlamydomonas reinhardtii* targeting signal of ribulose 1,5-bisphosphate carboxylase/oxygenase supported import into yeast mitochondria [1]. This report can be explained by use of yeast mitochondria that do not have to distinguish between mitochondrial and chloroplast precursors. Additionally, this targeting signal came from an algal source, which may have different signals for mitochondrial targeting based on the limited information available [2,3]. The reports of dual targeting of a number of proteins raises the question of how targeting specificity is maintained for the several hundred precursor proteins that are targeted to either organelle in higher plants [4–7].

Several studies have reported the specificity of import into mitochondria and chloroplasts using both in vitro and in vivo systems. In fact only one report of mis-targeting exists between plant mitochondria and chloroplasts. This is for the triose phosphate translocator of the chloroplast inner envelope membrane which is targeted to mitochondria in vitro, but not in vivo [8]. It should be noted that the mechanisms of

insertion of envelope proteins into chloroplast membranes are poorly understood, and thus this protein does not represent a typical chloroplast precursor [9]. Although in vivo systems have the advantage of reflecting an intact cellular system, such approaches are limited by the fact that they ignore the possible role of mature regions of the protein in sorting between organelles, and do not uncover mechanisms involved. In vitro approaches overcome these limitations but suffer from the fact that any sorting factors that may exist in the cell may not be present. To date the role of mature regions has been shown to be important for targeting to mitochondria and chloroplasts [10–12]. An extreme example is that of the carrier family of proteins on the inner mitochondrial membrane that contain internal targeting signals [13]. Despite initial reports of so-called 'targeting factors' it appears that such factors seem to be nascent chain associating proteins and play no role in targeting specificity [14,15].

In studying sorting specificity the terms dual, mis- and by-pass targeting are useful. Dual targeting describes a protein that is targeted to both organelles and has activity that can be detected in that location. Mis-targeting describes a protein targeted to an organelle where it has no function, or would not accumulate in vivo. By-pass targeting is a term that describes the low efficiency import of proteins into an organelle that occurs via by-passing the receptors on that organelle surface [16]. It is critical to distinguish between all these possibilities when studying a particular protein, as such a distinction will lead to more knowledge.

In this study we show that *Arabidopsis* ferrochelatase-I (Fc-I) and ferrochelatase-II (Fc-II) cannot be targeted to *Arabidopsis* mitochondria, despite the fact that such mitochondria can import a variety of mitochondrial specific and dual targeted proteins. The previous report of Fc-I targeting to mitochondria is due to the use of pea mitochondria, which appear to import a variety of chloroplast precursor proteins [17]. The use of appropriate controls when carrying out in vitro import assays can readily distinguish mis-targeting. Past reports in the literature describing a ferrochelatase activity in plant mitochondria must be re-evaluated as the complete genome sequence of *Arabidopsis* does not appear to contain a ferrochelatase that is mitochondrially targeted.

2. Materials and methods

2.1. Plant materials
Soybean (*Glycine max* [L] Merr. cv. Stevens) and Pea (*Pisum sativum* [L] Greenfeast) plants were grown in an environmentally controlled incubator at 28°C. The incubator was fitted with artificial

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lights of 600 μmol^{-2} s^{-1} set to a 16 h light and 8 h dark period. *Arabidopsis thaliana* plants were grown at 22°C with a 16 h light and 8 h dark photoperiod.

2.2. Mitochondrial and chloroplast isolation

Mitochondria were isolated from 7 day old soybean cotyledons and 10 day old pea leaves immediately upon harvest using the method of Day et al. [18]. Mitochondria were isolated from 7 day old *Arabidopsis* cell culture using a method based on Day et al. [18] with the following modifications. *Arabidopsis* cell suspension was ground with acid purified sand (BDH Chemicals, Vic., Australia) in a mortar and pestle. After isolation, crude mitochondria were layered on top of a discontinuous Percoll gradient composed of 5 ml 40%, 20 ml 21% and 10 ml 16% (v/v) Percoll containing 0.6 M mannitol, 20 mM TES and 2% (w/v) BSA. All other centrifugation steps were as for isolation of soybean and pea mitochondria. 10 day old pea leaf chloroplasts were isolated following published procedures, taking precautions to avoid any breakdown of chloroplast receptors as described [19,20].

2.3. In vitro protein import

Arabidopsis Fc-I and -II were amplified using cDNA isolated from 10 day old *Arabidopsis* plants, with the following primers: Fc-I forward, 5′-ATG CAG GCA ACG GCT TTA TC-3′; Fc-I reverse, 5′-CTA TAG GTT CCG GAA CGC ATG-3′; Fc-II forward, 5′-ATG AAT TGC CCA GCC ATG AC-3′; Fc-II reverse, 5′-TTA TAA TGA AGG CAAGAT GCC CC-3′. Precursor proteins for *Arabidopsis* Fc-I and Fc-II, soybean alternative oxidase (AOX), pea small subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase (SSU) and glutathione reductase (GR) were produced using the rabbit reticulocyte $T_N T^®$ in vitro transcription/translation kit (Promega, Madison, WI, USA) as described previously [21]. Mitochondrial and chloroplast imports were carried out as described previously, and imported proteins detected using phosphor-imaging as outlined [22]. Chimeric proteins containing the targeting signal of Fc-I linked to the mature proteins of GR and SSU were made using standard cloning techniques. This entailed taking the first 87 amino acids of Fc-I and linking it to the mature proteins. All constructs were confirmed by sequencing using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit and analyzed using an ABI 310 genetic analyzer according to manufacturer's instructions (Applied Biosystems, Melbourne, Australia).

2.4. Western blot

Western blot analysis of the mitochondrial proteins was carried out using 30 μg of mitochondrial protein, which was resolved by SDS–PAGE and transferred to a TransBlot® nitrocellulose membrane (Bio-Rad, Sydney, Australia) using a semi-dry blotting apparatus (Millipore, Sydney, Australia). Mitochondria were probed with antibodies to the outer membrane protein Tom20 (from Dr. H.-P. Braun). The bands were detected using chemiluminescence (Roche, Sydney, Australia) and visualized using a LAS 1000 (Fuji, Japan). The Western blots were analyzed with the Image Gauge v3.0 software (Fuji, Japan).

2.5. Enzyme assays

The outer membrane integrity of mitochondria was determined using the cytochrome *c* oxidation integrity assay. 10 μg of purified mitochondria were mixed with 1 ml of reaction media in an oxygen electrode chamber. To this, 20 μl of 0.5 M ascorbate and 10 μl of 2.5 mM cytochrome *c* were added and oxygen consumption measured. 10 μl of Triton X-100 (10% (v/v)) was added and the oxygen consumption measured. Cytochrome oxidase activity was inhibited by 10 μl of 0.1 M KCN. The percent of mitochondria with intact outer membranes was calculated by using the equation:

$$[1 - (\text{rate}^{+\text{cytochrome } c} - \text{rate}^{+\text{ascorbate}})/(\text{rate}^{+\text{Triton X-100}} - \text{rate}^{+\text{KCN}})] \times 100.$$

2.6. Arabidopsis genome search for additional ferrochelatase genes

Two genes, *Fc-I* (GenBank accession number X73417) and *Fc-II* (GenBank accession number Y13156) have been reported to encode ferrochelatase proteins from *Arabidopsis* which display 69% homology. A genome database search was performed to determine whether there were additional genes encoding ferrochelatase proteins. The homology search program tblastn [23] was used to search the *Arabidopsis* genome and only these two genes were found.

3. Results

3.1. Import of precursor proteins into mitochondria and chloroplasts

As part of a program to understand the nature of dual targeting signals we investigated the import of various dual targeted proteins into pea chloroplasts and mitochondria isolated from various plant tissues. *Arabidopsis* Fc-I has previously been reported to be dual targeted and thus we examined its import into mitochondria and chloroplasts [17]. We used the extensively studied AOX from soybean as a mitochondrial control [24], SSU from pea as a chloroplast control [25], and GR from pea as a control for dual targeting [26]. We examined import into *Arabidopsis* mitochondria, pea mitochondria and chloroplasts and soybean mitochondria. We found that *Arabidopsis* Fc-I was not imported into *Arabidopsis* or soybean mitochondria, despite the fact that these mitochondria clearly imported both the mitochondrial protein AOX, and the dual targeted protein GR, but not the chloroplast protein SSU as expected (Fig. 1). Thus these mitochondria displayed the predicted specificity for import. In contrast, pea leaf mitochondria did import *Arabidopsis* Fc-I, based on the generation of a processed product upon incubation with mitochondria that was not present in the translation mixture alone and was resistant to digestion by externally added protease (Fig. 1A, lanes 2 and 3). The precursor of Fc-I and the additional product present in the translation mixture were readily digested by externally added protease. However, these mitochondria also displayed import of SSU (Fig. 1A, lanes 2 and 3). This import was as efficient as the import observed for the authentic mitochondrial precursors. Efficiency was judged as the amount of protein imported compared to that added. Thus this import could not be defined as by-pass on the basis that it was efficient [16].

As there have been some reports of ferrochelatase activity in plant mitochondria, albeit some dating from over 30 years ago [27–29], we tested the import of Fc-II into mitochondria. No import was observed into *Arabidopsis*, pea or soybean mitochondria (Fig. 1A–C). Both Fc-I and Fc-II were imported into chloroplasts, which did not import the mitochondrial protein AOX (Fig. 1D). Searches of the *Arabidopsis* genome sequence revealed that there were no additional ferrochelatase sequences in the genome. Given that the mature Fc-I and Fc-II proteins display 69% homology, and high similarity is seen across species, it is unlikely that an additional ferrochelatase gene exists that cannot be detected with homology searches.

3.2. Intactness of mitochondrial isolations

Before ascribing the ability of pea mitochondria to import SSU and Fc-I as either mis- or by-pass targeting we determined the intactness of the outer membrane of the various mitochondrial preparations. As judged by cytochrome *c* latency assays, mitochondria from pea, *Arabidopsis* and soybean were judged to be 91%, 97% and 96% intact, respectively. Thus the import of SSU and Fc-I into pea leaf mitochondria could not be due to the fact that the outer membrane was ruptured and that these proteins could by-pass the outer membrane barrier. Furthermore we carried out Western blot analysis on these mitochondria with antibodies to Tom20, an exposed protein on the outer membrane which acts as a receptor for a variety of imported proteins [13,30]. We observed that Tom20 was present in all isolated
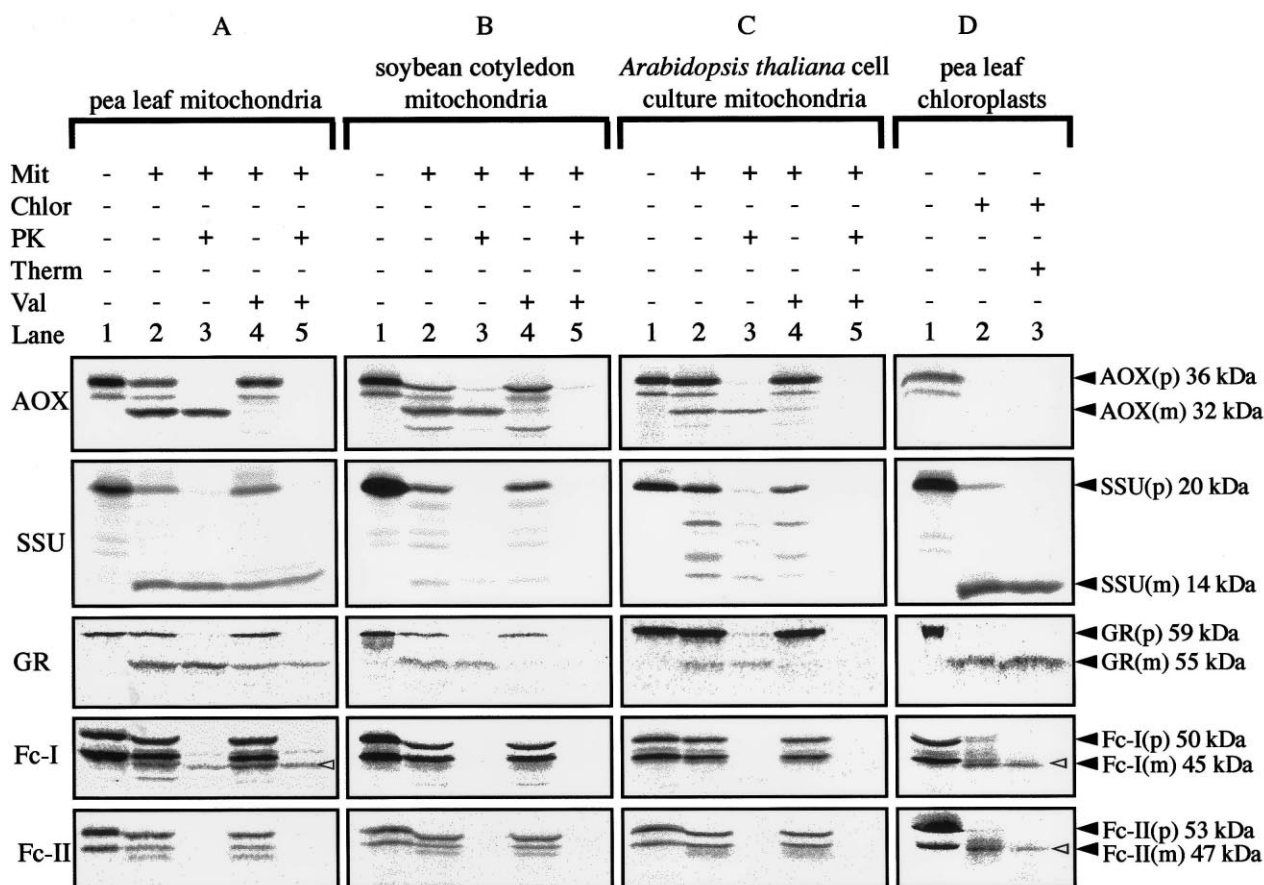
Fig. 1. Import of precursor proteins into isolated mitochondria and chloroplasts. In vitro import of precursor proteins AOX, SSU, GR, Fc-I and Fc-II was performed into (A) pea leaf mitochondria, (B) soybean cotyledon mitochondria, (C) *Arabidopsis thaliana* cell culture mitochondria, and (D) pea leaf chloroplasts. The precursor (p) and mature (m) forms of the proteins are indicated by arrows, with the apparent molecular weight of the proteins. A–C: Lane 1, precursor protein only. Lane 2, precursor protein incubated with mitochondria. Lane 3, lane 2 with PK added. Lane 4, lane 2 with valinomycin added. Lane 5, lane 4 with PK added. D: Lane 1, precursor protein only. Lane 2, precursor protein incubated with chloroplasts. Lane 3, lane 2 with thermolysin added. For clarity, open arrows indicate the mature ferrochelatase proteins. Abbreviations: Mit = mitochondria, Chlor = chloroplast, PK = proteinase K, Therm = thermolysin, Val = valinomycin, AOX = alternative oxidase, SSU = small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase, GR = glutathione reductase, Fc-I = ferrochelatase-I, Fc-II = ferrochelatase-II.

mitochondria (Fig. 2). These results confirm that the import of SSU and Fc-I could not be due to outer membrane damage of pea mitochondria, compared to that in soybean and *Arabidopsis* which did not display such import activities.

3.3. Targeting ability of the Fc-I transit peptide

We next investigated the targeting properties of the Fc-I transit peptide by making a series of chimeric constructs to determine if it could support the targeting of other passenger proteins to mitochondria. The targeting signal of Fc-I could not support mitochondrial import of either the dual targeted protein GR or SSU into mitochondria from pea, soybean or *Arabidopsis* (Fig. 3A–C). However it could support the import of these proteins into chloroplasts (Fig. 3D). Additionally, the targeting signal of Fc-I could not support the import of AOX into any mitochondrial preparation (data not shown). Thus we concluded that the targeting signal of Fc-I does not have mitochondrial targeting activity, but can support import of passenger proteins into chloroplasts.

4. Discussion

We have re-evaluated the import of Fc-I, previously re-

ported to be imported into mitochondria and chloroplasts and termed a dual targeted protein [17]. No import of Fc-I or passenger proteins could be detected into *Arabidopsis* or soybean mitochondria. Import of Fc-I into pea mitochondria could be detected but noticeably the targeting signal of Fc-I could not support import of any passenger proteins into pea mitochondria. The import of Fc-I into pea mitochondria was not due to the fact that the outer membrane was not intact and protein receptors not present. As the import of Fc-I and SSU into pea mitochondria was efficient it could not be ascribed to by-pass import. Thus we concluded that pea mitochondria allowed the mis-targeting of Fc-I and SSU. It was notable that this was only a property of pea mitochondria, as soybean and *Arabidopsis* did not display this activity, nor did mitochondria from potato, cowpea or soybean nodule mitochondria (Whelan – unpublished data). Additionally, yeast mitochondria that did import the dual targeted protein GR, and not the chloroplast protein SSU from pea, also did not import Fc-I (Whelan – data not shown). Previously we have observed the import of other chloroplast proteins such as Rubisco activase into pea mitochondria (Whelan and Chew, personal communication), and others have observed the import of plastocyanin into pea mitochondria [31].
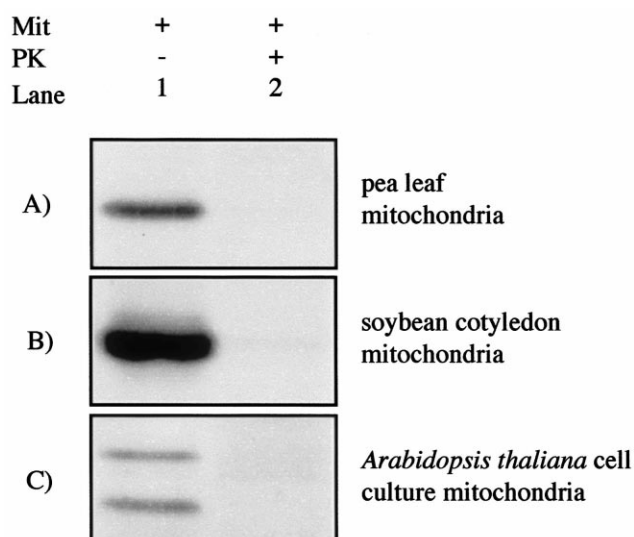
Fig. 2. Digestion of mitochondrial outer membrane protein Tom20. Mitochondria isolated from (A) pea leaves, (B) soybean cotyledons, and (C) *Arabidopsis thaliana* cell culture were incubated with 32 μg/ml PK for 30 min on ice. Proteins from both untreated and protease treated mitochondria were separated by SDS–PAGE. Immunodetection with an antibody for Tom20 was performed after transfer of the proteins onto a nitrocellulose membrane. Lane 1, untreated mitochondria. Lane 2, mitochondria incubated with PK. Abbreviations: Tom20 = 20 kDa translocase of the outer mitochondrial membrane.

We ascribe the targeting of Fc-I and other chloroplast proteins to pea mitochondria as mis-targeting. This is because it cannot be explained by a by-pass mechanism. The outer membrane is intact and exposed protein receptors on the outer membrane are present on pea mitochondria. In fact we can demonstrate that the import of SSU into pea mitochondria is dependent on a protein component on the outer mitochon-

drial membrane, as pre-treatment of these mitochondria with protease prevents the observed SSU import (Whelan and Chew, personal communication). This mis-targeting is easily detected using some simple controls. Firstly, this import is not dependent on a membrane potential. Such import is only possible if the protein does not enter into or across the inner mitochondrial membrane. However the reported ferrochelatase activity of plant mitochondria is reported to be associated with the mitochondrial inner membrane. Secondly, the selectivity of mitochondria can be tested using chloroplast precursors. When such precursors are seen to be efficiently imported into mitochondria, it is not a suitable system to assess dual import. Finally, the targeting signal of Fc-I does not support import of any passenger protein into mitochondria.

The finding that pea mitochondria are not a suitable system to assess targeting between mitochondria and chloroplasts has important implications. Firstly, pea mitochondria cannot be used to ascribe an organellar location to a protein that may be present in mitochondria and chloroplasts. As peas are widely used to study and isolate chloroplasts, it is crucial not to use pea mitochondria to determine protein targeting to mitochondria. Although it is desirable to use homologous systems as much as possible, the use of soybean or cowpea is superior, as both legumes are closely related to pea, are easy to isolate mitochondria from, and the mitochondria isolated display the predicted import specificity. Also of note was that the Fc-I targeted to pea mitochondria was from a rabbit reticulocyte translation lysate. In contrast, neither the mitochondrial proteins tested, nor the dual targeted protein GR were targeted to any mitochondria translated using a wheat germ lysate. Thus mis-targeting cannot be ascribed to the use of the translation lysate. However pea mitochondria can now be used as an assay system to determine how specificity is maintained, if indeed it is with these mitochondria. Thus reports of dual targeting and mitochondrial import activity with pea mitochondria need to be re-evaluated.
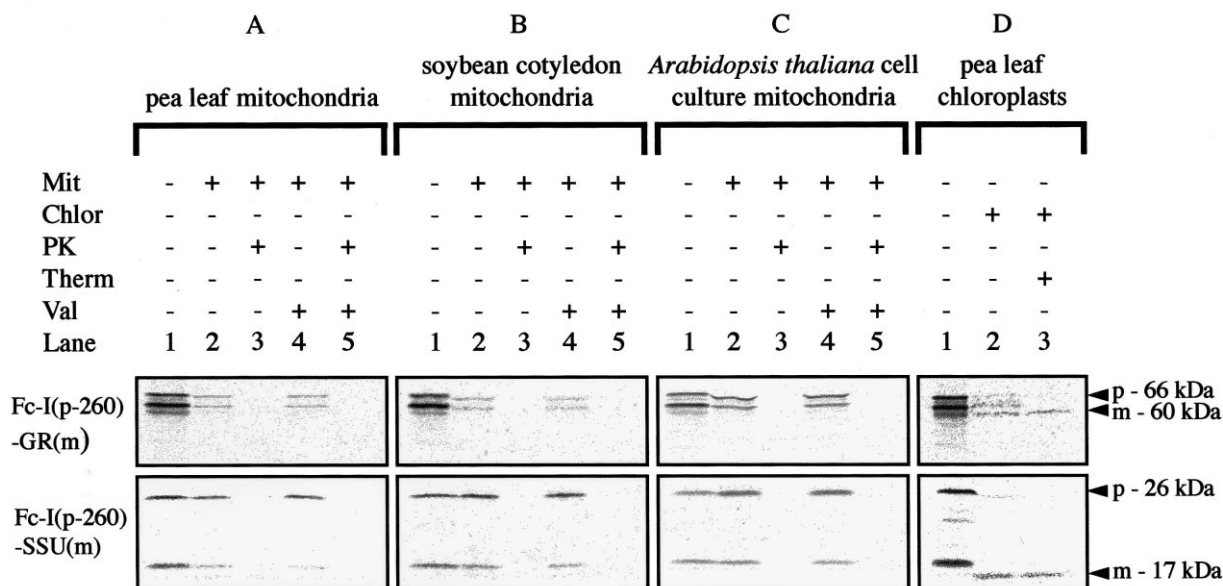

Fig. 3. Import of chimeric precursor proteins into isolated mitochondria and chloroplasts. In vitro import of chimeric precursor proteins Fc-I(p-260)–GR(m) and Fc-I(p-260)–SSU(m) was performed into (A) pea leaf mitochondria, (B) soybean cotyledon mitochondria, (C) *Arabidopsis thaliana* cell culture mitochondria, and (D) pea leaf chloroplasts. The precursor (p) and mature (m) forms of the proteins are indicated by arrows, with the apparent molecular weight of the proteins. A–C: Lane 1, precursor protein only. Lane 2, precursor protein incubated with mitochondria. Lane 3, lane 2 with PK added. Lane 4, lane 2 with valinomycin added. Lane 5, lane 4 with PK added. D: Lane 1, precursor protein only. Lane 2, precursor protein incubated with chloroplasts. Lane 3, lane 2 with thermolysin added. Abbreviations as for Fig. 1.

The finding that *Arabidopsis* Fc-I or Fc-II cannot be targeted to *Arabidopsis* mitochondria raises the question of the association of ferrochelatase activity in mitochondria. Ferrochelatase activity is clearly present in yeast and mammalian mitochondria, in the matrix but associated with the inner membrane ([32] and references therein). Ferrochelatase presence in plant mitochondria is less well defined as no immunological or purified activity data exist from plant mitochondria [29]. Its association with plant mitochondria is based on a small number of reports of mitochondria purified from etiolated tissue [28]. Unless very careful measures are taken, such mitochondria will contain non-green plastid contamination. Contamination needs to be carefully assayed for by monitoring the presence of either galactolipids or carotenoids, sensitive markers for plastid contamination [33,34]. Careful analysis of the reports of a plant mitochondrial localization for ferrochelatase indicates that sufficient measures to exclude all non-green plastids have not been undertaken. The initial report of ferrochelatase in plant mitochondria used only a crude mitochondrial pellet from potato obtained by differential centrifugation and thus contamination from either plastids (leucoplasts) or plasma membrane was likely [27]. Studies using etiolated barley identified the mitochondrial fraction on a sucrose gradient using succinate dehydrogenase and cytochrome oxidase activities. The presence of ferrochelatase in this fraction was taken to indicate a mitochondrial location for ferrochelatase, even though no contamination was assessed [28]. Likewise, a more recent study used etiolated barley but did not check for etioplast or plasma membrane contamination, which they reported also had ferrochelatase activity [35].

It is possible that heme for plant mitochondrial proteins is synthesized in plastids and exported to mitochondria. In yeast all iron–sulfur proteins are assembled inside the mitochondrion and then distributed to various locations within the cell [36]. Synthesis of heme only within plastids would have the added advantage of close coordination between mitochondrial and plastid activities via common regulation. Expression of yeast ferrochelatase that had the mitochondrial targeting sequence removed indicated that they were viable, and contained mitochondrial heme proteins [37]. Therefore mitochondrially located ferrochelatase does not appear to be an absolute requirement for cell viability. Alternatively either Fc-I or Fc-II may be targeted to the mitochondrion but by means other than a dual targeting presequence. Alternative splicing or differential start sites for transcription may produce altered forms of a protein that may be capable of being imported into mitochondria (and plastids?) [4]. However definite localization of ferrochelatase in plant mitochondria free from plastid contamination using either activity, immunological or proteomic approaches need to first demonstrate the presence of ferrochelatase activity in plant mitochondria, before the problem of targeting or sorting can be addressed. A recently described intact mitochondrial functional assay in yeast may provide a useful system for investigating the location of ferrochelatase activity in plant mitochondria [32].

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