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Import of precursor proteins into mitochondria from soybean tissues during development

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Abstract Characterisation of the amount of protein import of the alternative oxidase (AOX) and the F_Ad precursor proteins (previously shown to use different import pathways) into mitochondria from developing soybean tissues indicated that they displayed different patterns. Import of the AOX declined in both cotyledon and root mitochondria with increasing age, whereas the import of the F_Ad into cotyledon mitochondria remained high throughout the same period. Using primary leaf mitochondria, it was evident that import of AOX remained high while it declined in cotyledon and root mitochondria. The amount of import of the AOX into mitochondria from different tissues closely matched the amount of the Tom 20 receptor.

Key words: Mitochondrial protein import; Development; Soybean; Import pathway

1. Introduction

The yeast genome sequencing project indicates that there may be more than 500 proteins present in the mitochondrion. It is likely that a similar or greater number will be present in the mitochondrion from higher plants and animals. Given the tissue specialisation evident in higher organisms, the total number of mitochondrial proteins in higher organisms may be over 1000 [1]. Even in plants where the mitochondrial genome ranges in sizes from 200 to 2000 kb [2,3], still only a handful of proteins are encoded in the mitochondria while the remainder must be imported from cytosolically synthesised precursors [4].

A combination of biochemical and genetic approaches has given a great deal of information about the protein import process into mitochondria [5]. Even though studies on yeast (*Saccharomyces cerevisiae*) and *Neurospora crassa* have traditionally led the way, much information on plant and animal mitochondrial import systems has emerged in recent years. Although studies in plants (and animals) have used the 'fungal' models as a basis, it is emerging that these organisms contain significant differences. The plant mitochondrial processing peptidase (MPP) is an integral part of the cytochrome bc_1 complex [4,6] although a matrix activity has also been reported [7]. The signals required for mitochondrial import seem to differ between plants and other organisms, mistargeting of proteins reported from studies with yeast often do not stand up when a homologous plant system is used [4].

Although there may be significant differences in the mitochondrial import process across phylogenetic groups, it is also evident that overall, similarities are present in all systems. The concept of the general import pathway emerged from fungal studies and a similar pathway is likely to exist in plants and animals. In this import pathway, precursor proteins synthesised in the cytosol bind to cytosolic factors, essential for import, which then interact with the mitochondrial import receptor Tom 20[1]. The presequence is translocated deeper into the Tom complex via an acid-chain, i.e. it is exposed to higher affinity binding sites [5]. Tom 22 appears to play a central role in directing preproteins to the general insertion pore [10]. The precursor is passed from the Tom complex to the Tim complex where the presequence is translocated across the inner membrane. A membrane potential ($\Delta\psi$) is necessary to stabilise the presequence on the inside of the inner membrane before the translocation machinery pulls the protein into the mitochondrion. Subsequently processing, sorting and assembly takes place [5,11].

Mitochondrion protein import is considered a constitutive process. The proteins required for the import of a precursor protein are generally considered to be present to support mitochondrial import. The regulation of protein import into plant mitochondria has been reported. Although several studies reported that purified tobacco mitochondria did not import proteins [12–14], it was subsequently shown that this was due to the fact that import into isolated tobacco mitochondria occurs in a rhythmic manner [15]. Developmental regulation of protein import has been reported in pea leaf, which was proposed to be due to a reduction in the mtHSP 70 [16]. In animals, regulation of protein import has also been reported. Import of the mammalian cytochrome P450 was found to be tissue specific [17]. Thyroid hormone was shown to increase the import of certain precursor proteins, which was accompanied by the increase in components of the import apparatus [18].

Studies on protein import into plant mitochondria have revealed that several import pathways exist [4]. Tom 20 from potato appears to be imported into the outer membrane with the need for a protein receptor [19]. A general import pathway consisting of cytosolic factors, the Tom 20 receptor, Tim components and MPP have been characterised and precursor proteins such as the alternative oxidase (AOX) and $F_1\beta$ subunit of the ATP synthase appear to utilise this pathway in plants [4,5]. An additional pathway using a different receptor

[1] Uniform nomenclature for components of the mitochondrial import apparatus will be used [8,9], Tom = translocase of the outer membrane, Tim = translocase of the inner membrane, MPP = mitochondrial processing peptidase.

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has been characterised for the F_Ad subunit of the ATP synthase (F_Ad). This subunit does not require extramitochondrial ATP for import [20]. Other import pathways are likely to emerge with further studies, such as a pathway that may be responsible for the targeting of proteins to both mitochondria and plastids. Several such proteins have been classified into this group [21].

Here, we report studies involving the import of the AOX and F_Ad precursor proteins into mitochondria from developing soybean tissues. As these proteins have been shown to utilise different import pathways, we were interested if both pathways were regulated in the same manner in different tissues. We compared the amount of in vitro imported protein to the amount of various proteins present in mitochondria.

2. Material and methods

2.1. Plant materials

Soybean plants (*Glycine max* [L] Merr. cv. Stevens) were grown in an environmentally controlled incubator at 28°C. The incubator was fitted with artificial lights of 60 $\mu mol^{-2}\ s^{-1}$ set to a 16 h light and 8 h dark period.

2.2. Mitochondrial isolation

Soybean cotyledons and roots where harvested at 6, 8, 10, 12 and 16 days after germination. Cotyledon mitochondria were isolated from the tissues immediately upon harvest using the method of Day et al. [22]. For soybean root mitochondria, the homogenisation was carried out using a mortar and pestle, the homogenate was centrifuged at $2400\times g$ for 5 min and the supernatant was further centrifuged at $17\,400\times g$ for 20 min. Mitochondria from primary leaves were prepared 8 and 16 days after germination in a similar procedure for soybean cotyledon mitochondria.

2.3. In vitro protein import

Synthesis of the AOX (AOX 1) [23] and the F_Ad precursor proteins was carried out using the rabbit reticulocyte $T_NT^{®}$ in vitro transcription/translation kit (Promega, Madison, WI, USA) as described previously [24]. Import reactions where carried out with 5 mM succinate used as a substrate. To ensure no variation within a set (cotyledon and root mitochondria from all stages outlined above) of import experiments, a batch of precursor proteins was translated for use in all experiments within each set of imports. Import studies were carried out with a valinomycin control to ensure that all proteinase K (PK)-protected proteins were a result of import into or across the inner mitochondrial membrane. Quantification of the imports was carried out by determining the amount of imported protein using a BAS 2500 and imaging plate and calculating the amount of imported protein as pixel density per 100 μg of mitochondrial protein [15]. A dilution series of both precursor proteins was carried out to ensure linearity of detection, using the same imaging plates and exposure times. Quantification of imports was determined by densitometric analysis of raw image scans using the MacBas v2.0 imaging software (Fuji). Imported protein was defined as the amount of signal detected in the PK-treated lane, with the proviso that no signal was detected in the similarly treated valinomycin import reaction. Protein concentration was determined by the Lowry method [25]. When the import results are presented in table format, the highest amount of import is set to 100%, and other values expressed relative.

2.4. Western blot

Western blot analysis of the mitochondrial protein composition was carried out on all sets of mitochondria used for the import reactions. 30 μg of mitochondrial protein was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to TransBlot® nitrocellulose membrane (Bio-Rad, Sydney, Australia) using a semi-dry blotting apparatus (Millipore, Sydney, Australia). Mitochondria were probed with antibodies to the AOX (from Dr T.E. Elthon), subunit I of the cytochrome oxidase (COX I), (Molecular Probes, Eugene, OR, USA), ATP synthase (from Prof. E, Glaser), HSP 70 (from Prof. E. Glaser), HSP 60 (StressGen, Canada), cytochrome bc_1 (from Dr H.-P. Braun), TOM 20 (from Dr H.-P. Braun). The bands were detected using chemiluminescence (Roche, Sydney, Australia) and visualised using a LAS 1000 (Fuji). The Western blots were quantitated using the Image Gauge v3.0 software (Fuji) with the highest intensity band denoted a value of 100% and the remaining bands calculated relative to that value. Dilutions of mitochondria were carried out and probed with all the antibodies used to ensure linearity of detection for each antibody used.

3. Results

3.1. Import of the AOX and F_Ad during development

Import of the AOX and F_Ad precursor proteins was investigated into soybean root and cotyledon mitochondria during development. Import reactions were carried out with a valinomycin control (Fig. 1, lanes 4 and 5, 8 and 9), to ensure that the PK-protected products represent imported proteins. The AOX precursor protein with an apparent mol mass of 36 kDa was imported and processed to produce a mature product of 32 kDa (Fig. 1A), while the F_Ad precursor protein of 28 kDa was processed to produce a doublet of 21 and 20 kDa (Fig. 1B, represented by ○ and ●). The doublet mature product for the F_Ad was evident early in development and more prominent with root mitochondria. Fig. 1 represents a qualitative representation of import over development for the two tissues. It was evident that differences in the amount of import were occurring and we compared the amount of import of both these proteins on a mitochondrial protein basis.

Quantification of the import of AOX indicated that the amount of import declined during development in both tissues (Fig. 2A). In the case of cotyledon mitochondria, import

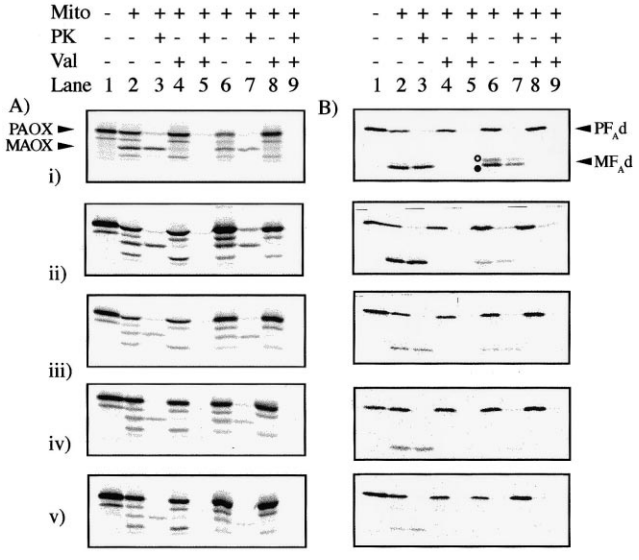

Fig. 1. Import of the AOX (A) and F_Ad subunit of the ATP synthase (B) into isolated soybean mitochondria of different ages. Each panel represents a different age, i = 6 days, ii = 8 days, iii = 10 days, iv = 12 days and v = 16 days. Lane 1, precursor protein alone, either AOX (PAOX) or F_Ad (PF_Ad). Lane 2, precursor protein incubated with purified soybean cotyledon mitochondria, a mature form of AOX (MAOX) and F_Ad (MF_Ad) are indicated. Lane 3, as lane 2 with PK added after the import reaction. Lane 4, as lane 2 with valinomycin added prior to the commencement of the import assay. Lane 5, as lane 4 with PK added. Lanes 6–9 are as lanes 2–5 except that purified soybean root mitochondria were used in the import assay. The open and closed circles indicate the doublet mature form produced for the F_Ad protein as discussed in the text.

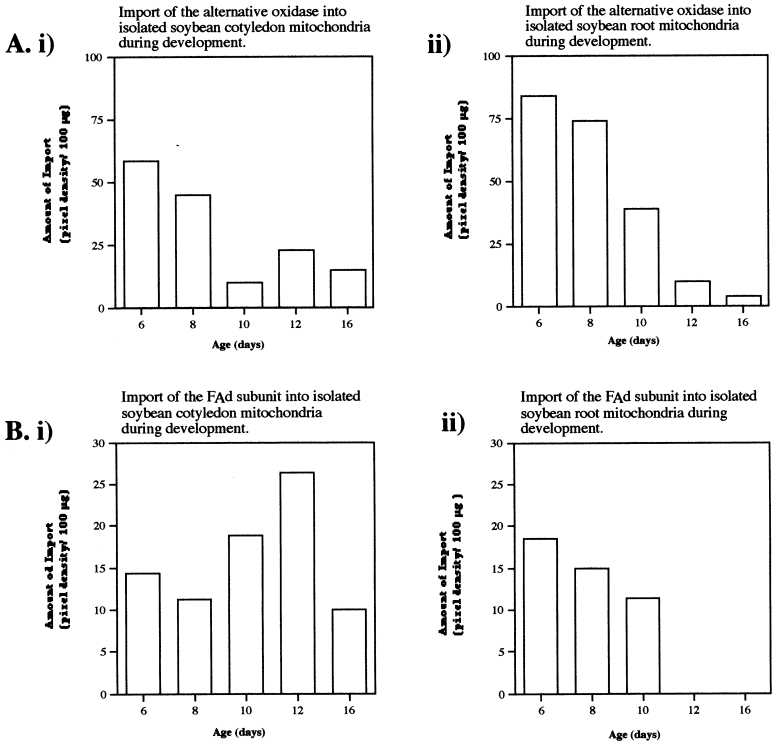

Fig. 2. Quantification of the amount of import into mitochondria from different tissues of different ages. The amount of import is indicated on the *y*-axis and the age of tissue on the *x*-axis. A, i = AOX import into cotyledon mitochondria; A, ii = AOX import into root mitochondria. B as A except that the F_Ad precursor was used in the import assays.

dropped 4-fold between day 6 and day 16. This decline in import was initially higher, in that from day 6 to day 10 greater than a 6-fold decline was observed. However, import increased slightly after day 10, so that overall a 4-fold decline in import was observed between the two end points (Table 1). A similar trend was seen with the import of AOX into root mitochondria except that the decline was greater (Fig. 2A, ii).

A different pattern was observed when the import of the F_Ad precursor protein was quantified (Fig. 2B). It was evident with cotyledon mitochondria that the import of this precursor did not decline as did the AOX, in fact import of the F_Ad precursor was higher at days 10 and 12 than at day 6. With root mitochondria, only a small decline was observed between day 6 and day 10 but after day 10, no import of the F_Ad was observed into root mitochondria. This is in contrast to cotyledon mitochondria where import was highest at day 12 and still evident at day 16 (Fig. 2B, ii).

To confirm that the overall pattern observed in the import studies was representative of the trend in import during cotyledon and root development, we repeated these studies several times. As the translation efficiency of each precursor can vary between batches of lysate, the different studies are presented individually in Table 1. We present three sets of data, set 1 is the results presented in Figs. 1 and 2, while the other sets are repeats carried out under similar conditions. It is evident from the data presented that the trend outlined in Fig. 2 was obtained in these studies. The import of AOX declined 4-fold or greater into soybean cotyledon and root mitochondria. In contrast, the import of the F_Ad precursor only declined 2-fold in cotyledon mitochondria and notably was highest at day 10 when import of the AOX had already decreased 4-fold. In root mitochondria, import of the F_Ad was only observed at days 6, 8 and 10, the latter approximately half that observed at the earlier time point. We have carried out other time points other than that shown here. If we isolate mitochondria earlier than 6 days, import can be initially lower, and then rise to a peak at 6 days for cotyledon mitochondria and decrease as shown here for the AOX precursor. Likewise, import declines to very low levels with older tissues [26].

We investigated if the import pathway for AOX could still be high in some tissues while declining in other tissues. This was possible using soybean because primary leaves are developed sufficiently at 8 days after germination to prepare mitochondria. Analysis of import of AOX into cotyledon, root

Table 1
The amount of import of AOX and F_Ad into soybean cotyledon and root mitochondria from three separate experiments

		Set 1			Set 2			Set 3		
Age (days)		6	10	16	6	10	16	6	10	16
AOX	cots	100	16	25	100	21	19	100	21	7
	roots	100	46	5	100	58	25	100	36	27
F_Ad	cots	76	100	53	100	89	41	70	100	50
	roots	100	61	0	100	40	0	100	67	0

The maximum amount of import for each set was normalised to 100% and other values expressed relative.

A)

Tissue\Age	8	16
Cots	100	30
Roots	100	9
Leaves	100	66

B)

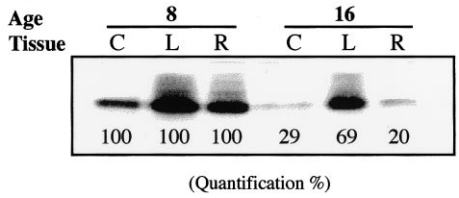

Fig. 3. Amount of import of AOX (A) and Western blot analysis (B) using antibodies against Tom 20 to probe mitochondrial proteins isolated from 8 and 16 day old cotyledons, leaves and roots. The highest amount of import or Tom 20 detected for each tissue (on day 8) was set to 100% and other values expressed relative.

and leaf mitochondria at 8 and 16 days indicated that while import had declined over 3-fold in cotyledon mitochondria and 10-fold in root mitochondria between days 8 and 16, import remained high in leaf mitochondria showing only a 30% decline on a mitochondrial protein basis (Fig. 3A). We concluded from this that even though the import pathway for AOX declined steadily in cotyledon and root mitochondria, it remained high in leaf mitochondria.

Therefore, a contrasting trend was evident in import between precursor proteins previously shown to use different import pathways. The import of the AOX precursor declined in both cotyledon and root mitochondria with increasing age. In contrast, import of the F_Ad was high when the import of the AOX precursor was very low. Import of the F_Ad precursor did decline in root mitochondria, but rather than a gradual decrease that was observed with the AOX, it was very dramatic in that no import was observed at later stages. Higher levels of import of AOX were observed into leaf mitochondria, when import of this precursor had declined several fold in both cotyledon and root mitochondria.

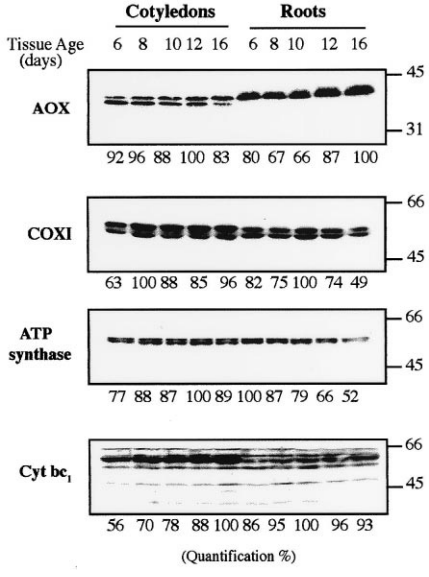

Fig. 4. Western blot analysis of respiratory chain components of mitochondria used in the import assays. The maximum amount of protein for each tissue was set to 100% and other values expressed relative. The size of mol mass markers in kDa indicated on the left was used to determine the apparent mol mass of the bands detected. Abbreviations outlined in Section 2.

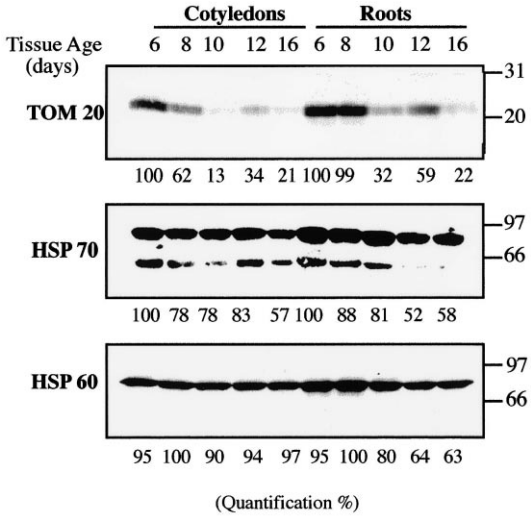

Fig. 5. As Fig. 4 except that antibodies to components of the import apparatus were used to probe mitochondrial samples.

3.2. Mitochondrial proteins during development

3.2.1. Respiratory chain proteins. In order to understand how the changes in import correspond to the level of mitochondrial proteins, we carried out Western blot analysis for several mitochondrial proteins to investigate if any and to what extent protein composition was changing. Previous studies with developing soybean cotyledons and root mitochondria have extensively characterised changes in respiration occurring in these systems [27,28].

Characterisation of respiratory chain proteins indicated that the changes in the amount of proteins did not correspond to changes in import. For AOX, two isoforms were detected in cotyledon mitochondria (Fig. 4), corresponding to AOX 2 (34 kDa) and AOX 3 (36 kDa) [29]. The amount shown for the AOX is for both proteins, which do not change dramatically over development. However, AOX 2 does decrease and AOX 3 increases, so even though total AOX remains largely unchanged, AOX 3 increased in abundance while the general import pathway was decreasing. In root mitochondria, AOX 3 is the only isoform detected, which increases slightly at the later stages. In the case of cytochrome oxidase, the amount of COX I (52 kDa, mitochondrially encoded) was characterised. We routinely detect a doublet for this subunits on Western blot analysis. In cotyledon mitochondria, COX I increased slightly between day 6 to day 8 and remained relatively constant thereafter (Fig. 4). In root mitochondria, an increase was also observed between day 6 and day 10 and decreased by 50% at day 16 (Fig. 4). Analysis of the ATP synthase complex showed a similar trend to that of COX I. Two prominent bands were detected of 55 and 56 kDa, corresponding to the α and β subunits of the ATP synthase, although we cannot assign which band is which for soybean [30]. In the case of the cytochrome bc_1 complex, the core proteins of 47, 52 and 53 kDa were detected. Given that an additional isoform has

been reported for each, it is not unexpected that four distinct bands can be detected [31]. In cotyledon mitochondria, the amount of these proteins rises almost 50% from day 6 to 16. In root mitochondria, the levels of these proteins remain relatively constant throughout the development period examined (Fig. 4).

In conclusion, it appears that the amounts of respiratory proteins detected are in close agreement with previous studies. In cotyledon mitochondria, the amount of proteins rises a little and remains constant thereafter, while in root mitochondria, a decrease at later stages, especially day 16, was evident.

3.2.2. Import components. Analysis of the amount of import components was carried out by Western blot analysis to determine if they changed during the development period. Tom 20, the outer membrane receptor for the general import pathway, was detected with antibodies to the potato Tom 20 [19]. In cotyledon mitochondria, it was evident that the amount of Tom 20 declined quite sharply from a maximum at day 6 to a minimum at day 10, followed by a slight rise (Fig. 5). The overall decrease in Tom 20 correlated closely with the trend in import seen for the AOX into cotyledon mitochondria outlined in Fig. 2. With root mitochondria, a similar pattern was observed except that the decline was not as dramatic as seen in cotyledons, again similar to what was evident with the trend in import. However, there was a small increase at day 12, which was not accompanied by an increase in import into root mitochondria (Fig. 5). Examination of the amount of Tom 20 in primary leaf mitochondria where import of AOX remains high at day 16, but has declined several fold in cotyledon and root mitochondria, indicated that the amount of Tom 20 correlated closely with the amount of import observed in this tissue (Fig. 3).

In the case of mtHSP 70, a different pattern was observed, although a decline of almost 50% was observed over the period examined for both root and cotyledon mitochondria (Fig. 5), this decline did not correspond in magnitude or temporally to the decline in import. For cotyledon mitochondria at day 12, HSP 70 was still $\sim$80% of the level detected at day 6 even though import had reach a minimum. In fact except for day 16, the level of HSP 70 was about 80% of the maximum detected, and even at day 16 it was almost 60%. In root mitochondria, a similar picture emerged except that the decline in HSP 70 took place at day 12, a little earlier than was detected in cotyledons (Fig. 5). Importantly, the decline in HSP 70 took place after the decline in import for both tissues, not coincident or before the decline in import.

In the case of HSP 60, a different picture, no change was detected in cotyledon mitochondria, but in root mitochondria a decline was observed at days 12 and 16 (Fig. 5).

In conclusion, from the examination of the import components it was evident that only the amount of Tom 20 correlated with the amount of import. Importantly, the magnitude of decline of Tom 20 correlated with the amount of import.

4. Discussion

An understanding of how proteins reach their functional location in a cell is essential for a complete understanding of cellular regulation. Research defining the components of targeting and import machinery has led to the identification of many proteins involved in this process. However, only a few reports exist on the regulation of the process of protein import, and mitochondrial protein import in particular, even though the process of protein targeting is clearly a prime target for regulation.

Examination of protein import into isolated mitochondria using two different precursor proteins from soybean tissues indicated that no overall pattern existed. In the case of AOX, a precursor protein that requires both extra- and intramitochondrial ATP for import, a decrease was evident in both cotyledon and root mitochondria with increasing age. In contrast, the F_Ad precursor, which does not require extramitochondrial ATP for import, did not decrease in a similar manner in cotyledon mitochondria, and in root mitochondria displayed abrupt changes. Comparison of the pattern of import observed with the two precursors indicated that while the import of AOX was very low (day 10 cotyledons), the import of F_Ad was near a maximum, and increasing compared to the previous time point. Therefore, any causative mechanism to account for a decrease in import of AOX must be compatible with the high amount of import of the F_Ad observed.

Extending these studies to primary leaves, it was evident that although a large decrease had taken place in root and cotyledon mitochondria, only a small decrease has taken place in leaf mitochondria. This decrease evident in leaves can be accounted for to some degree by a change in the amount of matrix to membrane components of mitochondria with increasing age. An increase in glycine decarboxylase in the mitochondrial matrix with developing leaves would mean that although the same amount of mitochondrial protein was present, less mitochondria, and thus less import components, would be present in older leaves [32]. We concluded that although mitochondrial import does decrease with development, this differs with different precursors and tissues.

In order to understand how the above changes relate to mitochondrial function, we carried out Western blot analysis with several mitochondrial proteins of the respiratory chain and components of the import apparatus. Traditionally, it has been extremely difficult to quantitate the amount of proteins using this approach, even using luminescence substrates quantitation has proved difficult due to the narrow linear range of X-ray film. Quantitation using such methods requires several dilutions of sample material, primary antibody and variation in exposure time. Even then, scanning of X-ray film is a subjective process. However, full quantitation of signal strength is possible with capture of luminescence signal using digital means.

Using such methods, it was evident with soybean cotyledon mitochondria that although some changes were evident with respiratory chain components, none of these changes corresponded to the changes in import observed, particularly to the decline in import observed with the AOX. Overall, there was some increase in proteins detected between day 6 and day 8 or 10 and afterwards only small changes were observed. With root mitochondria, a slightly different pattern was observed. While AOX and the cytochrome bc_1 complex did not change, an almost 50% decrease in the subunits of cytochrome oxidase and ATP synthase was detected. The decrease in these latter components is in close agreement with the activities previously reported in a developmental study of respiration in soybean roots [27,28].

To investigate the mechanistic basis for the pattern of import observed, we examined the levels of components of the import apparatus. Examination of the amount of HSP 70

indicated that it decreased during development. However, this decrease did not correspond in either magnitude or timing with any decrease in import observed. When import was at a minimum for AOX in cotyledon mitochondria at day 10, HSP 70 was still at 80%. In root mitochondria at day 12 when import had decreased by over 5-fold, only a 2-fold decrease in HSP 70 was evident. In fact, it was clear that the decrease in HSP 70 observed was after the decrease in import. Thus any decrease in HSP 70 observed may be the result of a decrease in import and not the cause. The fact that HSP 70 is not causing the decrease in the import of AOX is consistent with the fact that import of the F_Ad was still observed, which would require the action of mitochondrial HSP 70 for import as we have previously shown that it requires intramitochondrial ATP [20].

Examination of the amount of HSP 60 indicated that it remained largely unchanged in cotyledon mitochondria, whereas a decrease was evident in the latter two stages of root mitochondria. Again the changes did not correspond to the decrease in import observed with the AOX. In yeast, HSP 60 is imported in a similar pathway to soybean F_Ad, in that it does not require extramitochondrial ATP [33]. In soybean cotyledon mitochondria where no decrease in the import of the F_Ad was observed, no decrease in HSP 60 was observed. In root mitochondria where no import of F_Ad was observed at the last two stages, a decrease in the amount of HSP 60 was detected. This again suggests that the amount of import may dictate the amount of protein in the mitochondrion.

Examination of the amount of Tom 20 indicated that it correlated well with the amount of import of AOX observed. In cotyledon mitochondria, the amount of Tom 20 exactly correlated with the amount of AOX import detected. To investigate further the correlation of import of AOX and the amount of Tom 20, we investigated the amount of Tom 20 in leaf mitochondria, in addition to root and cotyledon mitochondria. Again a good correlation was observed with the amount of Tom 20 and the amount of import of AOX in leaf mitochondria. Although the amount of Tom 20 corresponds to the pattern of import of AOX, it does not mean that the amount of Tom 20 is the regulator of import. Other components of the Tom complex, or cytosolic factors necessary for import may play critical roles. The amount of Tom 20 may only reflect the amount of such factors.

It has previously been reported that the amount of HSP 70 controls the amount of import and this does not appear to be the case in the studies we carried out [16]. The differences may be accounted for by one or a combination of several factors. Our studies were carried out with isolated soybean mitochondria, whereas studies indicating HSP 70 was the limiting factor for import were carried out in pea. Therefore, the differences observed may be due to the fact that in different species (and tissue) mitochondrial import is controlled in a different manner. However, the magnitude and timing of the decrease in import observed with pea do not correspond to the magnitude and timing of the decrease in HSP 70. Therefore, as we observed it may be the decrease in import that causes the decrease in HSP 70 in pea.

Also in the studies outlined here we have measured the amount of import, i.e. protein import into mitochondria standardised on a mitochondrial protein basis. We also measured the efficiency of import, that is defined as the amount of imported protein divided by the amount of protein associated with the mitochondrion, expressed as a percentage value. A measure of the efficiency of import may indicate at which stage import was regulated. If binding to the outer membrane was limiting, then the efficiency of import would not change even if the amount decreased, as anything that did bind could be imported. However if the translocation stage of import was limited, as it would if mtHSP 70 was limiting, then the efficiency of import would decrease because binding could still occur, but less translocation from the bound state would take place. If the measure of efficiency is to be used, it presumes that all bound (associated with mitochondria?) protein can be imported. We have previously shown that we can get import from the bound state of both AOX and F_Ad, but only approximately 20–50% of such bound protein [20]. The reason for this is that much binding may be non-specific, or not receptor-mediated. Removing the presequence from both the AOX and F_Ad and carrying out import experiments indicated that they still bound, but no import was observed (unpublished data) [34]. Therefore, the efficiency of import cannot be accurately measured because of non-productive binding.

We have quantified the amount of import and the amount of various mitochondrial proteins using digital detection systems that have greater than three orders of magnitude in detection. We have also experimentally verified that the signals we detect are in the linear range for the precursors and antibodies used. In contrast, the study in pea used detection on X-ray film for both import and Western blot, and quantitation was carried out by subsequent scanning. Such detection methods are very difficult to quantify, and linearity depend on the type of film, correct pre-flashing, use of chemical enhancement agents and signal strength and exposure times within narrow limits [35]. Finally in our studies, we have used a 100% homologous system. We have used two precursors representing two different import pathways into three tissues. The three tissues are obtained from the same plant, harvested at the same time, so an overall picture is achieved.

In conclusion, the different pattern of import observed between precursors with various tissues indicates that protein import into mitochondria has the potential to be a global regulator of subsets of mitochondrial proteins in a tissue specific manner. Given that differential regulation of import pathways has also been observed in animals [18], it may indicate that mitochondrial protein import is an important regulatory mechanism in higher organisms from diverse phylogenetic groups.

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