Environmental stresses inhibit and stimulate different protein import pathways in plant mitochondria

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Received 14 May 2003; revised 6 June 2003; accepted 6 June 2003

First published online 19 June 2003

Edited by Ulf-Ingo Flügge

Abstract The impact of various environmental stresses (drought, chilling or herbicide treatment) on the capacity of plant mitochondria to import precursor proteins was investigated. Drought treatment stimulated import and processing of various precursor proteins via the general import pathway. The stimulatory effect of drought on the general import pathway was due to an increased rate of import, was accompanied by an increased rate of processing, and could be attributed to the presequence of the precursor protein. Interestingly, drought decreased the import of the F_Ad subunit of ATP synthase suggesting a bypass of the point of stimulation during import of this precursor. Both chilling and herbicide treatment of plants, on the other hand, caused inhibition of import with all precursors tested. No decrease in processing of imported proteins was observed by these stress treatments. Western analysis of several mitochondrial proteins indicated that the steady-state level of several mitochondrial components, including the TOM20 receptor and the core subunits of the cytochrome bc_1 complex responsible for processing, remained largely unchanged. Thus environmental stresses differentially affect import of precursor proteins in a complicated manner dependent on the import pathway utilised.

Key words: Environmental stress; Mitochondrial import; Processing; Import pathway

1. Introduction

Environmental stresses encompass a wide range of physical conditions, which can significantly alter plant metabolism, growth and development, leading at their extremes to plant death. These abiotic stresses include drought, high salinity, extremes of temperature (both high and low), heavy metal toxicity, ultraviolet radiation, nutrient deprivation, high light stress and hypoxia. Plants by their very nature, being embedded in the soil, are unable to escape exposure to these environmental extremes and therefore must respond to survive. Many abiotic stresses increase active oxygen species (AOS) in plants due to perturbations of metabolism, including that in chloroplasts and mitochondria. These AOS can act as important signal molecules and interact with other signalling molecules to achieve integrated responses to a variety of stress and developmental triggers [1–3]. These responses include activation of gene expression cascades such as those triggered by the binding of the DRE binding protein to the dehydration response (DRE/C-repeat) *cis*-elements during drought and low temperature stress [4,5]. Microarray studies of gene expression following environmental stress suggest that while some common gene responses are observed, the majority of expression changes are specific to the stress applied [6]. New proteins synthesised in response to these stresses must be targeted to various subcellular compartments in order to function and for a plant to achieve an appropriate response to stress. It is of interest, therefore, to examine the response of the protein import machinery of organelles to environmental stress.

Subcellular organelles such as mitochondria and plastids are not only potential targets for damage during stress but may also play an important role in stress response. A range of mitochondrial responses to oxidative stress have been observed in plants. Major changes in over 30 different mitochondrial proteins have been observed following direct oxidative stress of *Arabidopsis* cells [7]. Inhibition of key metabolic proteins in plant mitochondria by the lipid peroxidation end-product, HNE, following AOS accumulation during various environmental stresses has also been documented [8]. Small heat shock proteins, some located in the mitochondrion, accumulate during oxidative stress [9], and, in some extreme cases, oxidative stress results in programmed cell death in *Arabidopsis* cells [10]. The effects of environmental stress on chloroplasts have been even more extensively studied, especially the means by which excess excitation energy is sensed, signalled and responded to [11]. The induction of various plastid-located forms of anti-oxidant enzymes, such as superoxide dismutase [12,13] and components of the glutathione and ascorbate cycles [14,15], indicates their involvement in the plastid's stress response. Proteomic approaches have shown the location of several other anti-oxidant enzymes in

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Abbreviations: AOS, active oxygen species; AOX, alternative oxidase; F_Ad, F_Ad subunit of mitochondrial ATP synthase; ANT, adenine nucleotide transporter; SSU, small subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase; GR, glutathione reductase; RSP10, nuclear encoded ribosomal protein 10; Tim23, translocase of the inner membrane protein 23

0014-5793/03/$22.00 © 2003 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)00691-4

the thylakoid lumen as well as proteins involved in proteolysis and refolding [16].

As the coding capacity of mitochondria and plastids is limited, many changes in their protein complement in response to environmental stress require that the proteins be imported from the cytosol where they are synthesised. We report here the first investigation of the effect of environmental stress on import of precursor proteins into plant mitochondria. We have used various precursor proteins that are known to utilise different import pathways into plant mitochondria: the alternative oxidase (AOX) as a precursor protein imported via the general import pathway [17], the F_Ad subunit of mitochondrial ATP synthase (F_Ad) as a precursor protein imported by a modified general import pathway that does not require extramitochondrial ATP [18] and the adenine nucleotide translocator (ANT) that enters via the carrier import pathway [19]. To ensure the fidelity of the import assay, we used a dual import assay that contains both mitochondria and chloroplasts and monitored any non-specific import into mitochondria using the precursor to the small subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase (SSU) [20].

2. Materials and methods

2.1. Materials

Tryquat 200 (Crop Care Australasia, Australia) is a commercially available herbicide combination of paraquat (437.5 mg/l) and diquat (225 mg/l). Studies on the mode of action of these two components appear to indicate that both function identically [21] and this combination is referred to here simply as paraquat. All other chemicals were at least reagent grade.

Pea (*Pisum sativum* L. cv. Green Feast) plants were germinated in vermiculite and grown in controlled environment chambers with a light intensity of 700 μmol/m^2/s, at 24°C and 65% humidity, for 10 days on a 16/8-h day/night cycle.

Precursor proteins were translated from soybean alternative oxidase (*Gm*AOX) [22], tobacco alternative oxidase (*Nt*AOX) [23], pea glutathione reductase (*Ps*GR), [24], soybean F_Ad (*Gm*F_Ad) [25], maize adenine nucleotide translocator (*Zm*ANT) [26], pea SSU (*Ps*SSU) [27] using the rabbit reticulate T_NT in vitro transcription/translation kit (Promega, Madison, WI, USA). *Arabidopsis thaliana* ribosomal protein S10 (*At*RSP10) was amplified using primers designed to sequences obtained from cDNA to the *Rsp10* gene (X80694) [28], and translated as described above. Precursor proteins were stored at −80°C and thawed on ice prior to use.

2.2. Abiotic treatments

Herbicide treated plants were sprayed evenly with paraquat (662.5 mg/l), allowed to dry and then returned to controlled environment chambers and exposed to light for 12 h until harvest. For low temperature treatment, plants were placed at 4°C for 36 h prior to harvest, whilst maintaining a normal day/night light cycle. For drought treatment, plants were not watered for 7 days prior to harvest whilst maintaining a normal humidity, temperature and day/night light cycle.

2.3. Pea mitochondrial isolation

Mitochondria were isolated according to the methods of Day et al. [29] and Zhang and Wiskich [30] from approximately 60 g of pea leaves. Leaves were disrupted with a Polytron (Kinematica, Kriens, Switzerland) in 250 ml of cold extraction medium (0.3 M sucrose, 25 mM tetrasodium pyrophosphate, 10 mM KH_2PO_4, 2 mM EDTA, 1 mM glycine, 1% (w/v) PVP-40, 1% (w/v) bovine serum albumin (BSA), 20 mM ascorbate, pH 7.5). The homogenate was filtered through four layers of miracloth and centrifuged for 5 min at 1100×*g*. The supernatant was centrifuged for 20 min at 18 000×*g* and the pellet resuspended in 200 ml of wash medium (0.3 M sucrose, 10 mM TES (*N*-trishydroxymethyl-2-aminoethanesulphonic acid), 1 mM glycine, 0.1% (w/v) BSA, pH 7.5) and centrifuged for 5 min at 1100×*g*. The supernatant was centrifuged for 20 min at 18 000×*g* and the pellet resuspended in 10 ml of wash medium, 5-ml aliquots were then layered over 27.5 ml solution (0.3 M sucrose, 10 mM TES, 1 mM glycine, 0.1% (w/v) BSA, 28% (v/v) Percoll and a linear gradient of 0–10% (w/v) PVP-40, pH 7.5) in a centrifuge tube, and centrifuged for 40 min at 40 000×*g*. The mitochondria were found as a tight light yellow-brown band near the bottom of the tube. The mitochondrial fraction was removed and diluted in 250 ml of wash medium and centrifuged at 31 000×*g* for 15 min. The supernatant was removed and this wash was repeated, the final mitochondrial pellet was resuspended in approximately 1 ml of wash medium and kept on ice until use.

2.4. Chloroplast isolation

Chloroplasts were isolated from 10-day-old pea leaves as described by Bruce et al. [31] and Waegemann and Soll [32]. Approximately 50 g of pea leaves were harvested and homogenised in 200 ml of grinding medium (50 mM HEPES–KOH pH 7.3 (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulphonic acid]), 330 mM sorbitol, 0.1% BSA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA). The homogenate was then filtered through one layer of miracloth and centrifuged for 5 min at 2000×*g*. The pellet was resuspended in residual grinding medium and layered over a 50% continuous Percoll gradient and centrifuged for 20 min at 12 100×*g*. The chloroplast band was collected and centrifuged for 4 min at 2000×*g*. The pellet was resuspended in a small volume of wash buffer and kept on ice until use. The chlorophyll concentration was determined using the method of Arnon [33].

2.5. In vitro dual import of proteins

Import into mitochondria and chloroplasts using a dual import system was carried out as previously described by Rudhe et al. [20]. Mitochondria and chloroplasts were isolated separately and combined in a buffer supporting both mitochondrial and chloroplastic import (0.3 M sucrose, 15 mM HEPES–KOH pH 7.4, 5 mM KH_2PO_4, 0.2% BSA, 4 mM methionine, 4 mM ATP, 1 mM GTP, 0.2 mM ADP, 5 mM succinate, 4.5 mM dithiothreitol, 10 mM potassium acetate and 10 mM $NaHCO_3$) and incubated for 20 min at 25°C. Following incubation the reactions were placed on ice and divided into two aliquots, one containing 120 μg/ml thermolysin supplemented with 0.1 mM $CaCl_2$ and the other untreated, both of which were then incubated for 30 min on ice. The thermolysin activity was inhibited by the addition of 10 mM EDTA. Each sample was then loaded on a 4% Percoll gradient and centrifuged for 30 s at 4000×*g*. Mitochondria and chloroplast fractions were collected separately and recovered by centrifugation at 20 800×*g* and 800×*g*, respectively. Imported products were analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and visualised using a BAS2500 (Fuji, Tokyo, Japan). Specific proteins detected were quantitated using the Image Gauge v3.0 software (Fuji).

2.6. In vitro processing assay

The processing reaction was carried out as described previously [34]. The reaction for processing (total volume 20 μl) contained 1 μl of [^{35}S]methionine-labelled precursor protein in 0.5% Triton X-100, 30 mM Tris–HCl (tris[hydroxymethyl]aminoethane) (pH 8.0), 1 mM $MgCl_2$, 1 mM methionine and 30 μg of mitochondrial protein. The processing reaction was carried out in the presence of protease inhibitors, E64 and apyrase, which were incubated with mitochondria for 5 min on ice before the addition of precursor. Processing was carried out at 25°C for the times shown in the figure legends. The reactions were stopped by the addition of protein loading buffer and boiling at 95°C for 3 min. Processed products were analysed by SDS–PAGE and visualised using a BAS2500 (Fuji). Specific proteins detected were quantitated using the Image Gauge v3.0 software (Fuji). Negative controls containing 2 mM 1,10-phenanthroline were carried out during each reaction.

2.7. Protein analysis

Protein concentrations were determined by the method of Peterson [35] using BSA as standard. 20 μg of total mitochondrial protein was solubilised in sample buffer (0.125 M Tris–HCl, 4% (w/v) SDS, 15% (v/v) glycerol and 20% (v/v) mercaptoethanol) and separated under denaturing, reducing conditions on 0.1% (w/v) SDS 12% (w/v) polyacrylamide gels according to Laemmli [36]. For immunoreaction experiments, proteins were electroblotted from SDS–PAGE gels onto Hybond-C+ extra membrane (Amersham Pharmacia Biotech, Sydney, Australia) using a Hoefer Semi-Phor semi-dry blotting apparatus

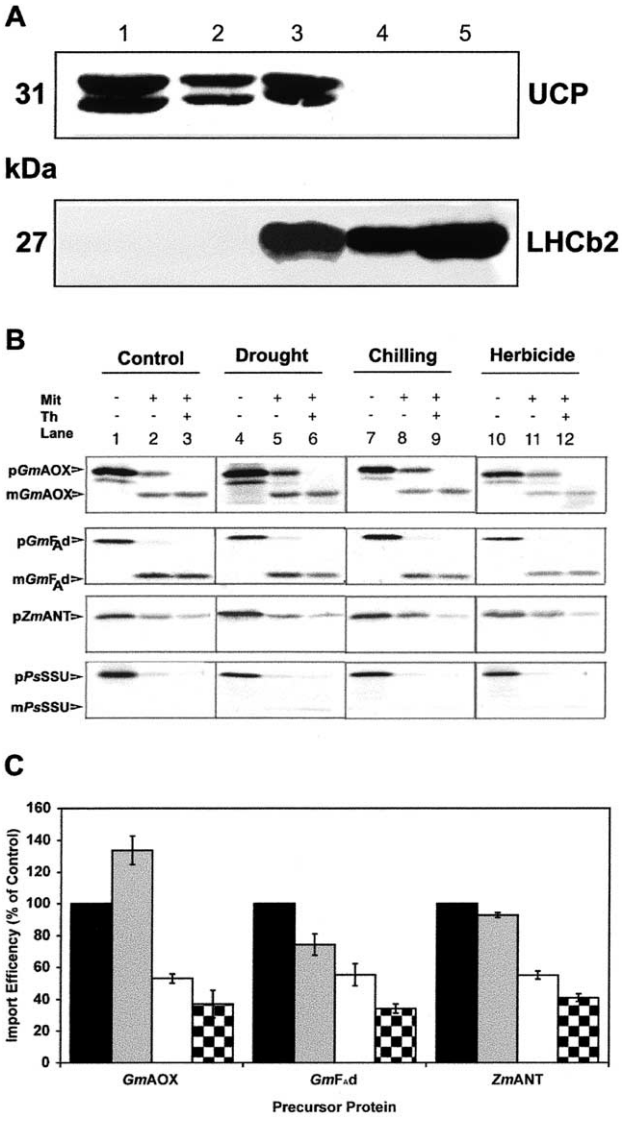

Fig. 1. The in vitro dual import system, and the import of *Gm*AOX, *Gm*F_Ad, *Zm*ANT and *Ps*SSU into mitochondria isolated from plants exposed to various environmental stresses. A: Western blot analysis of mitochondrial and chloroplastic fractions using UCP and LHCb2 antibodies. Lane 1, purified pea leaf mitochondria. Lane 2, purified pea leaf mitochondria that had been mixed with purified pea leaf chloroplasts and re-isolated. Lane 3, isolated pea leaf mitochondria mixed with isolated pea leaf chloroplasts. Lane 4, purified pea leaf chloroplasts that had been mixed with purified pea leaf mitochondria and re-isolated. Lane 5, purified pea leaf chloroplasts. B: Import of precursor proteins into mitochondria using a dual import system. Lane 1, precursor protein alone. Lane 2, precursor protein incubated with isolated pea leaf mitochondria. Lane 3, as lane 2 but with the addition of thermolysin. Lanes 4–6, 7–9 and 10–12 as lanes 1–3. C: The amount of import of protein precursors into plant mitochondria isolated from stressed pea plants, control (black), drought (grey), chilled (white) and paraquat-treated (checkerboard). Values are the average of three treatments (mean ± S.E.M.). For each precursor protein each stress is significantly different from control ($P < 0.05$) except for the import of ANT into mitochondria from drought-stressed plants.

(Amersham Pharmacia Biotech) and blocked. Mitochondrial proteins were probed with primary antibodies, 1/1000 dilution of anti-outer membrane receptor of 20 kDa (TOM20) serum, 1/2500 of anti-HSP70 serum, 1/2500 dilution of anti-HSP60 serum (Stress-Gen, Victoria, Canada), 1/10 000 dilution of anti-cytochrome bc_1 serum, 1/2000 dilution of anti-porin serum, a 1/10 000 dilution of an antibody to the mitochondrial inner membrane uncoupling protein (UCP) and a 1/5000 dilution of an anti-chloroplastic light-harvesting complex b2 subunit (LHCb2) serum [20]. Cross-reacting bands were detected by chemiluminescence of horseradish peroxidase-conjugated secondary antibodies (Roche, Sydney, Australia) and visualised using a LAS 1000 (Fuji). Specific proteins detected 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 band.

The cDNA encoding *At*Tom20-3 was prepared by reverse transcription polymerase chain reaction and cloned into the plasmid pQE60 (Qiagen®) for expression in *Escherichia coli*. Rabbit antiserum against the recombinant *At*Tom20-3 detects the ~20-kDa protein in whole cell extracts of *A. thaliana* and can immunoprecipitate *At*Tom20 from ^{35}S-labelled plants.

3. Results

3.1. Measurement of protein import into pea leaf mitochondria

Mitochondria isolated from the leaves of pea plants were utilised with in vitro assays to assess their ability to import precursor proteins. As isolated pea leaf mitochondria have previously been shown to import some chloroplast proteins, possibly via non-physiological mechanisms [37,38], we used a more stringent dual in vitro import assay which maintains the predicted organelle specificity and allows only mitochondrial precursors to be imported into mitochondria [20]. Purified mitochondria and chloroplasts were mixed together and precursor proteins added to the mixture. After treatment with externally added protease to remove any unimported protein, the organelles were separated and the amount of import into mitochondria assessed by autoradiography. The purity of the re-isolated mitochondria was demonstrated by their lack of cross-reactivity with antiserum to the chloroplast marker LHCb2, while strong reactivity with an antibody raised to the mitochondrial UCP was evident (Fig. 1A, lane 2) [20]. When mitochondria and chloroplasts were combined, both products were clearly detected (Fig. 1A, lane 3).

3.2. Stresses affect import pathways differentially

Pea plants were grown for 10 days and subjected to either cold, drought or herbicide application, treatments that have been shown previously to induce various degrees of oxidative stress and damage mitochondrial proteins [8]. In vitro import assays into mitochondria isolated from these plants yielded

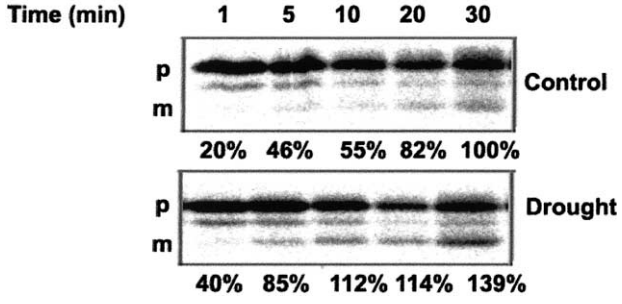

Fig. 2. Processing of *Gm*AOX with lysed mitochondria. Mitochondria were isolated from control and drought-treated pea leaves. Isolated mitochondria were lysed and incubated with *Gm*AOX in a buffer that supports processing. Aliquots were removed at different time points and the reactions were immediately stopped by the addition of sample buffer and boiling. Numbers below the blots represent the average ($n = 3$) percentage intensity of band relative to the 30-min time point of control mitochondria.

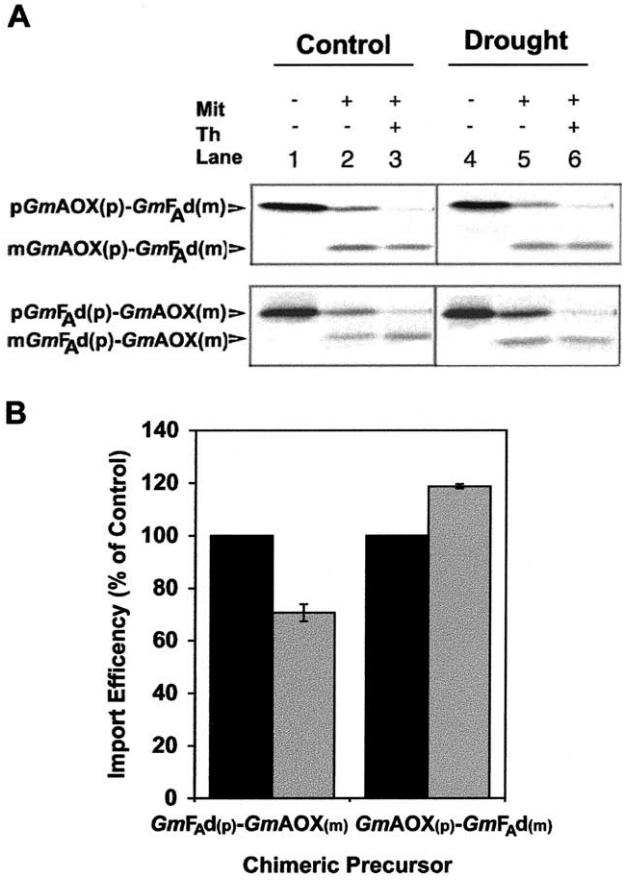

Fig. 3. The effects of drought on the amount of import of chimeric precursors, $GmF_Ad_{(p)}$-$AOX_{(m)}$ and $AOX_{(p)}$-$F_Ad_{(m)}$. A: Import of chimeric constructs into mitochondria using a dual import system. Lane 1, chimeric constructs alone. Lane 2, precursor protein incubated with isolated pea leaf mitochondria. Lane 3, as lane 2 but with the addition of thermolysin. Lanes 4–6 as lanes 1–3. B: Mitochondria were isolated from drought-stressed (grey) and control (black) pea plants. Values are the average of three treatments (mean ± S.E.M.). The import of both chimeric precursors was significantly different from control ($P < 0.05$) during drought stress.

consistent changes in import ability, depending on the stress and the precursor protein examined (Fig. 1B,C). Herbicide treatment caused >60% inhibition of import with all of the precursor proteins, while chilling stress inhibited import by approximately 50%. Induced water deficit, on the other hand, differentially affected the import of different precursor proteins. Import of *Gm*AOX into mitochondria from drought-treated plants was stimulated approximately 20% while that of GmF_Ad was inhibited by at least 20%. Import of the *Zm*ANT precursor protein was only marginally affected (5% inhibited) by drought treatment (Fig. 1B,C).

Examination of the import profile of the precursor proteins revealed no gross differences in binding and processing by the various treatments (Fig. 1B). However, as a previous report on mammalian mitochondria showed that inhibition of protein import during oxidative stress was due to decreased processing of precursor proteins, we investigated this aspect in more detail. In vitro processing assays using mitochondria from drought-treated plants indicated that processing was also stimulated (Fig. 2). In contrast, the decrease in import with chilling and herbicide treatment was not accompanied by a decrease in processing (data not shown).

3.3. Drought stress acts on the presequence and increases the rate of import

In order to investigate the differential effect of drought treatment on the import of *Gm*AOX and GmF_Ad precursor proteins in more detail, we made chimeric precursor proteins which contained the presequence of AOX linked to the F_Ad mature protein [$AOX_{(p)}$-$F_Ad_{(m)}$] and the presequence of F_Ad linked to the AOX mature protein [$F_Ad_{(p)}$-$AOX_{(m)}$] (see [39]). When we carried out import assays with these hybrid precursor proteins, we again observed a 20% stimulation of import with the *Gm*AOX presequence and a decrease in import with the GmF_Ad presequence, regardless of the structural protein which they preceded (Fig. 3). This shows that the differential response of the two precursors to drought treatment was due to the different properties of their presequences. We have already shown that these two precursor proteins interact with different protease-sensitive components on the outer mitochondrial membrane [18].

The effect of drought on import of other mitochondrial precursor proteins which use the general import pathway was examined: *At*RSP10, *Ps*GR and *Nt*AOX. Each of these precursor proteins contained a cleavable presequence and required intra- and extra-mitochondrial ATP for import. All of them displayed a significant and consistent increase in import

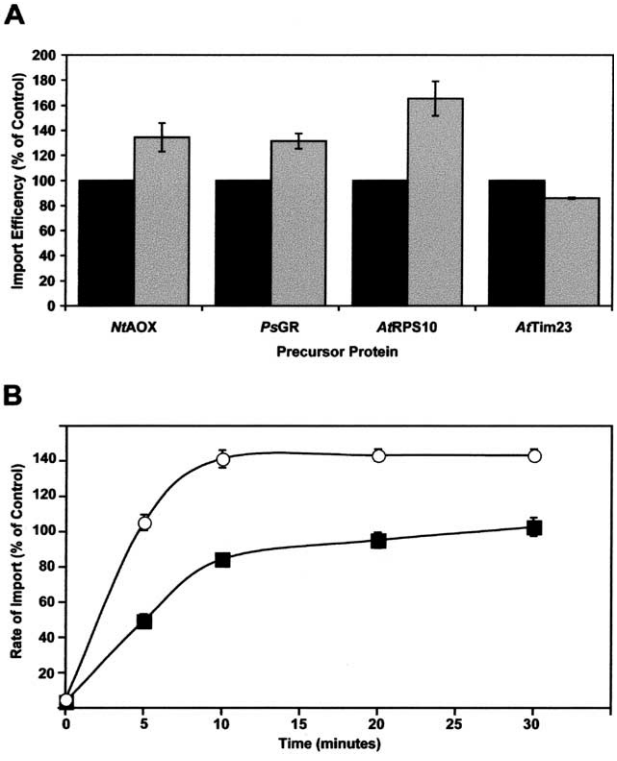

Fig. 4. Effect of environmental stresses on the amount of import of the mitochondrial precursors, *Nt*AOX, *Ps*GR, *At*RSP10 and *At*-Tim23 (A) and a comparison of the rate of import of *Gm*AOX into mitochondria isolated from drought-treated and control pea plants (B). A: Mitochondria were isolated from drought-stressed (grey) and control (black) pea plants. Values are the average of three treatments (mean ± S.E.M.). The import of all precursor proteins was significantly different from control ($P < 0.05$) during drought stress. B: Mitochondria were isolated from drought-stressed (○) and control (■) pea plants. Values are the average of three treatments (mean ± S.E.M.). 100% import represents the amount of import after 30 min into control mitochondria and other time points were expressed relative to this point.

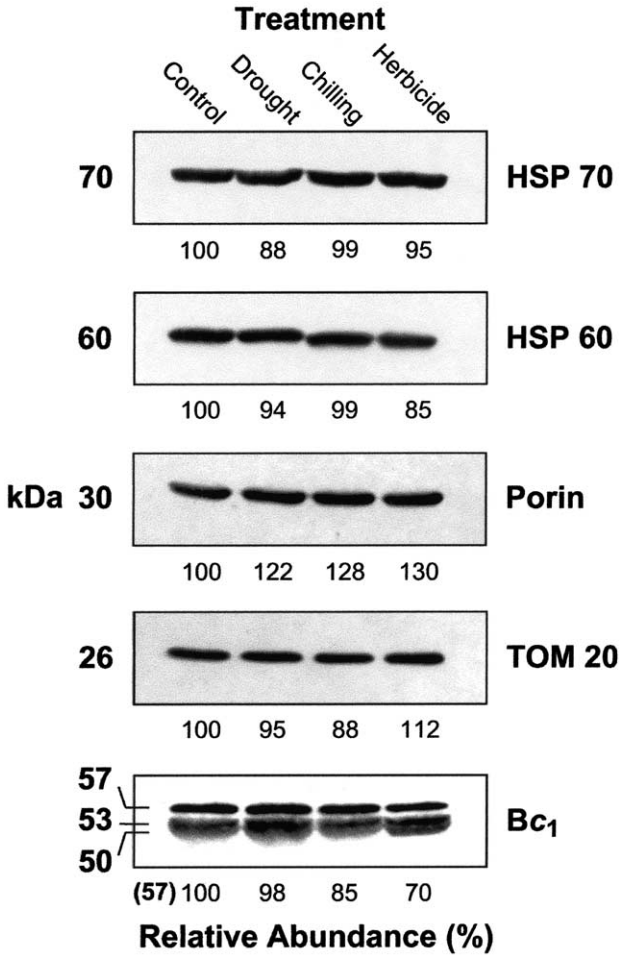

Fig. 5. Effect of environmental stresses on mitochondrial components. Mitochondria were isolated from stress-treated pea plants and their proteins separated by SDS–PAGE. Numbers on the left represent apparent molecular masses in kDa and those below the blots, the percentage intensity of band relative to the control. The HSP70 (70 kDa) panel is HSP70 protein detected using an anti-HSP70 antibody. The HSP60 (60 kDa) panel is the HSP60 protein detected with an anti-HSP60 antibody. The 30-kDa band in the Porin panel are porin proteins detected with the anti-porin antibody. The TOM20 (26 kDa) panel is the TOM20 proteins detected using an anti-TOM20 antibody. The 57-, 53- and 50-kDa bands in the bc_1 panel are bc_1 proteins detected with the anti-bc_1 antibody (the 57-kDa band was quantified).

of 35–50% (Fig. 4A). In contrast, the translocase of the inner membrane protein 23 (*At*Tim23), which, like *Zm*ANT, uses the distinct carrier import pathway [40], was slightly inhibited by drought (Fig. 4B) [19,20]. We conclude, therefore, that the stimulation of import of some proteins by drought was due to a specific effect of this treatment on the general import pathway.

It has been shown previously that plant mitochondrial import is linear for the first 10–15 min of the assay [18,41]. After this period, little or no increase in import is observed and the imported processed protein is stable in mitochondria for 30 min or more [41,42]. Consequently, we usually carry out in vitro assays for a period of 20 min. To investigate if the increased amount of import observed with mitochondria isolated from drought-treated plants was due to an increased rate of import, or a more sustained linear phase, imported products were sampled at various time points in a 30-min assay (Fig. 4B). It was clear from this analysis that the initial rate of import was increased in mitochondria from drought-treated plants compared to control (almost two-fold over the first 5 min of the assay).

3.4. Analysis of mitochondrial components during stress

Western blotting against several different mitochondrial proteins was performed to investigate if the changes in import outlined above coincided with changes in mitochondrial protein abundance. Analysis of the outer membrane component porin indicated that it increased slightly in all the stress treatments, suggesting no general loss in outer membrane following stress treatments. Analysis of a key component of the import machinery, TOM20, showed that its abundance was not changed by the stress treatments. 2D-PAGE analysis confirmed that there was no change in relative abundance of the different TOM20 isoforms present in pea leaf mitochondria (data not shown) [43]. The core subunits of the cytochrome bc_1 complex, which are responsible for removal of presequences during import of proteins, were not affected significantly. Nor were the amounts of both HSP70 and HSP60. We have previously reported that AOX protein abundance increased dramatically in all of the stress treatments [8].

4. Discussion

We have analysed the import of several different precursor proteins that use different import pathways, into mitochondria isolated from plants treated with various environmental stresses. Previously it has been shown that these stresses damage a number of mitochondrial proteins by modification of lipoic acid moieties, resulting in loss of enzyme activity [8]. All three stresses also induced the synthesis of AOX of the respiratory chain [8]. However, these same stresses have differential effects on protein import, depending on the nature of the precursor protein and the pathways used. Chilling and herbicide inhibited import of proteins via all three of the main import routes known to operate in plant mitochondria (Figs. 1 and 4). Drought treatment, on the other hand, while also inhibiting import via the F_Ad and the carrier import pathways, stimulated import of precursor proteins using the general import pathway. Thus while different environmental stresses may lead to general oxidative damage in the cell, not all mitochondrial processes are equally affected. The stimulation of the general import pathway observed in drought-stressed plants could not be mimicked by decreasing the severity or duration of the other stresses, which simply reduced the degree of inhibition (data not shown). Drought stress has been shown to induce many more genes than chilling stress in a study which analysed the expression profiles of 7000 genes in *Arabidopsis* [6,44]. Thus drought may have effects on gene expression and protein function via processes other than oxidative stress, including, for example, osmotic changes in plant cells.

We have not yet identified a molecular site of action for either inhibition or stimulation of import (Fig. 5), but our data eliminate some possibilities and point to specific areas in the import process that could be involved. For example, the stimulatory effect of drought treatment appeared to involve the presequence of precursors using the general import pathway and increased the initial rate of uptake of the proteins (Figs. 3 and 4). Since all precursor proteins use a single Tom

complex and the same Tom40 pore [45–47], it appears that the different effects on the different import pathways occur prior to insertion into the Tom40 pore. Import of AOX and F_Ad has been previously shown to involve different protease-sensitive components on the mitochondrial surface for recognition [18], and it is possible that the component specific to the general import pathway is stimulated by drought. However, this is not the only component affected since the stimulation of import was accompanied by a similar increase in processing activity of the imported protein. We utilised a variety of precursor proteins from various plant species, including pea, in this study. All these precursor proteins were imported into control mitochondria and processed to the same apparent mol mass as judged to be the correct mature form from previous studies characterising the import of these proteins. We concluded from this that they followed authentic import pathways into mitochondria and that similar results would be observed with a wide variety of precursor proteins irrespective of their origin. The effects observed were on the import pathways, not on the individual precursor proteins.

Operation of the import process is central to mitochondrial biogenesis both to repair damaged organelles and to increase mitochondrial bulk during regrowth. Ensuring the fidelity of the process during stress may be important to tolerance of stresses such as drought and would assist in recovery of plant growth.

Acknowledgements: We thank the following colleagues for their gifts of antibodies: Dr T.E. Elthon, Department of Biological Sciences, University of Nebraska, Lincoln, NE, USA, for anti-porin antibodies; Dr Hans-Peter Braun, Institut für Angewandte Genetik, Universität Hannover, Hannover, Germany, for the anti-bc_1 antibodies; and Dr Iwona Adamska, Stockholm University, Stockholm, Sweden, for the LHCb2 antibody. This work was supported by Australian Research Council Grants to J.W., D.A.D., T.L. and A.H.M. and the Swedish Foundation for International Co-operation in Research and Higher Education Grants to E.G. and J.W. N.L.T. acknowledges a University Postgraduate Scholarship from the University of Western Australia.

References

[1] Dat, J., Vandenabeele, S., Vranova, E., Van Montagu, M., Inze, D. and Van Breusegem, F. (2000) Cell. Mol. Life Sci. 57, 779–795.
[2] Vranova, E., Inze, D. and Van Breusegem, F. (2002) J. Exp. Bot. 53, 1227–1236.
[3] Hoeberichts, F.A. and Woltering, E.J. (2003) BioEssays 25, 47–57.
[4] Shinozaki, K. and Yamaguchi-Shinozaki, K. (2000) Curr. Opin. Plant Biol. 3, 217–223.
[5] Cushman, J.C. and Bohnert, H.J. (2000) Curr. Opin. Plant Biol. 3, 117–124.
[6] Seki, M. et al. (2002) Plant J. 31, 279–292.
[7] Sweetlove, L.J., Heazlewood, J.L., Herald, V., Holtzapffel, R., Day, D.A., Leaver, C.J. and Millar, A.H. (2002) Plant J. 32, 891–904.
[8] Taylor, N.L., Day, D.A. and Millar, A.H. (2002) J. Biol. Chem. 277, 42663–42668.
[9] Banzet, N., Richaud, C., Deveaux, Y., Kazmaier, M., Gagnon, J. and Triantaphylides, C. (1998) Plant J. 13, 519–527.
[10] Tiwari, B.S., Belenghi, B. and Levine, A. (2002) Plant Physiol. 128, 1271–1281.
[11] Mullineaux, P. and Karpinski, S. (2002) Curr. Opin. Plant Biol. 5, 43–48.
[12] Abarca, D., Roldan, M., Martin, M. and Sabater, B. (2001) J. Exp. Bot. 52, 1417–1425.
[13] Kurepa, J., Herouart, D., Van Montagu, M. and Inze, D. (1997) Plant Cell Physiol. 38, 463–470.
[14] Yabuta, Y., Motoki, T., Yoshimura, K., Takeda, T., Ishikawa, T. and Shigeoka, S. (2002) Plant J. 32, 915–925.
[15] Mittova, V., Guy, M., Tal, M. and Volokita, M. (2002) Free Radic. Res. 36, 195–202.
[16] Peltier, J.B. et al. (2002) Plant Cell 14, 211–236.
[17] Whelan, J., Hugosson, M., Glaser, E. and Day, D.A. (1995) Plant Mol. Biol. 27, 769–778.
[18] Dessi, P., Smith, M.K., Day, D.A. and Whelan, J. (1996) Arch. Biochem. Biophys. 335, 358–368.
[19] Lister, R., Mowday, B., Whelan, J. and Millar, A.H. (2002) Plant J. 30, 555–566.
[20] Rudhe, C., Chew, O., Whelan, J. and Glaser, E. (2002) Plant J. 30, 213–220.
[21] Dodge, A.D. (1971) Endeavour 30, 130–135.
[22] Whelan, J., McIntosh, L. and Day, D.A. (1993) Plant Physiol. 103, 1481.
[23] Whelan, J., Smith, M.K., Meijer, M., Yu, J.W., Badger, M.R., Price, G.D. and Day, D.A. (1995) Plant Physiol. 107, 1469–1470.
[24] Creissen, G., Edwards, E.A., Enard, C., Wellburn, A. and Mullineaux, P. (1992) Plant J. 2, 129–131.
[25] Smith, M.K., Day, D.A. and Whelan, J. (1994) Arch. Biochem. Biophys. 313, 235–240.
[26] Bathgate, B., Baker, A. and Leaver, C.J. (1989) Eur. J. Biochem. 183, 303–310.
[27] Anderson, S. and Smith, S.M. (1986) Biochem. J. 240, 709–715.
[28] Adams, K.L., Daley, D.O., Whelan, J. and Palmer, J.D. (2002) Plant Cell 14, 931–943.
[29] Day, D.A., Neuburger, M. and Douce, R. (1985) Aust. J. Plant Physiol. 12, 219–228.
[30] Zhang, Q. and Wiskich, J.T. (1995) Arch. Biochem. Biophys. 320, 250–256.
[31] Bruce, B.D., Perry, S., Froelich, J. and Keegstra, K. (1994) in: Plant Molecular Biology Manual, 2nd edn., pp. J1–J15, Kluwer Academic, Dordrecht.
[32] Waegemann, K. and Soll, J. (1995) Methods Cell Biol. 50, 255–267.
[33] Arnon, I. (1949) Plant Physiol. 24, 1–15.
[34] Szigyarto, C., Dessi, P., Smith, M.K., Knorpp, C., Harmey, M.A., Day, D.A., Glaser, E. and Whelan, J. (1998) Plant Mol. Biol. 36, 171–181.
[35] Peterson, G.L. (1977) Anal. Biochem. 83, 346–356.
[36] Laemmli, U.K. (1970) Nature 227, 680–685.
[37] Lister, R., Chew, O., Rudhe, C., Lee, M.N. and Whelan, J. (2001) FEBS Lett. 506, 291–295.
[38] Cleary, S.P. et al. (2002) J. Biol. Chem. 277, 5562–5569.
[39] Tanudji, M., Dessi, P., Murcha, M. and Whelan, J. (2001) Plant Mol. Biol. 45, 317–325.
[40] Murcha, M.W., Lister, R., Ho, A.Y.Y. and Whelan, J. (2003) Plant Physiol. 131, 1737–1747.
[41] Stahl, A., Pavlov, P.F., Szigyarto, C. and Glaser, E. (2000) Biochem. J. 349, 703–707.
[42] Stahl, A., Moberg, P., Ytterberg, J., Panfilov, O., Brockenhuus Von Lowenhielm, H., Nilsson, F. and Glaser, E. (2002) J. Biol. Chem. 277, 41931–41939.
[43] Werhahn, W., Niemeyer, A., Jansch, L., Kruft, V.V., Schmitz, U.K. and Braun, H.P. (2001) Plant Physiol. 125, 943–954.
[44] Seki, M., Narusaka, M., Abe, H., Kasuga, M., Yamaguchi-Shinozaki, K., Carninci, P., Hayashizaki, Y. and Shinozaki, K. (2001) Plant Cell 13, 61–72.
[45] Neupert, W. (1997) Annu. Rev. Biochem. 66, 863–917.
[46] Rehling, P., Wiedemann, N., Pfanner, N. and Truscott, K.N. (2001) Crit. Rev. Biochem. Mol. Biol. 36, 291–336.
[47] Ahting, U., Thieffry, M., Engelhardt, H., Hegerl, R., Neupert, W. and Nussberger, S. (2001) J. Cell Biol. 153, 1151–1160.