Differential Impact of Environmental Stresses on the Pea Mitochondrial Proteome*®

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Exposure to adverse environmental conditions causes oxidative stress in many organisms, leading either to disease and debilitation or to response and tolerance. Mitochondria are a key site of oxidative stress and of cellular response and play important roles in cell survival. We analyzed the response of mitochondria in pea (Pisum sativum) plants to the common stresses associated with drought, cold, and herbicides. These treatments all altered photosynthetic and respiratory rates of pea leaves to various extents, but only herbicides significantly increased lipid peroxidation product accumulation. Mitochondria isolated from the stressed pea plants maintained their electron transport chain activity, but changes were evident in the abundance of uncoupling proteins, non-phosphorylating respiratory pathways, and oxidative modification of lipoic acid moieties on mitochondrial proteins. These data suggest that herbicide treatment placed a severe oxidative stress on mitochondria, whereas chilling and particularly drought were milder stresses. Detailed analysis of the soluble proteome of mitochondria by gel electrophoresis and mass spectrometry revealed differential degradation of key matrix enzymes during treatments with chilling being significantly more damaging than drought. Differential induction of heat shock proteins and specific losses of other proteins illustrated the diversity of response to these stresses at the protein level. Cross-species matching was required for mass spectrometry identification of nine proteins because only a limited number of pea cDNAs have been sequenced, and the full pea genome is not available. Blue-native separation of intact respiratory chain complexes revealed little if any change in response to environmental stresses. Together these data suggest that although many of the molecular events identified by chemical stresses of mitochondria from a range of model eukaryotes are also apparent during environmental stress of plants, their extent and significance can vary substantially. Molecular & Cellular Proteomics 4:1122–1133, 2005.

Cellular homeostasis can be disrupted by changes in the extracellular environment that uncouple biochemical pathways, which normally operate under a range of biophysical and chemical constraints. Such disruption is often accompanied by the undesirable accumulation of metabolic intermediates, and the most studied of these are reactive oxygen species (ROS)1 produced by disruption of metabolism and electron transport chains. Much attention has focused on the impact of ROS during normal development and aging of cells and during perturbation of normal cellular life by disease, toxins, and the physical environment. Oxidative stress modification of proteins tends to increase with age in most organisms (1), but plants have recently been noted as an exception to the normal trend and are able to manage oxidative protein damage much more effectively during growth and later reproductive phases of life (2).

Mitochondria form a focus for much oxidative stress research as not only are they the sites of oxygen consumption and a significant source of cellular ROS, but oxidative damage of the organelles perturbs the cell’s energy supply required for repair mechanisms. Consequently the nature of oxidative damage to mitochondria is being investigated in a variety of organisms. These studies are providing information of the general susceptibilities of these organelles to damage as well as uncovering a range of defense mechanisms specific to experimental conditions and the mitochondrial protein profile found in different organisms. A series of studies have analyzed the detailed sites of protein oxidation in mitochondria from various organisms under control conditions and following in vitro oxidation protocols. These reveal that a complex array of oxidative modifications occur, including carbonyl group formation (3, 4), oxidation of tryptophan (5), tyrosine nitration (6), and oxidative modification of enzyme cofactors such as lipoic acid (7). Concomitant with imposed oxidative damage, specific proteins are either synthesized or lost from mitochondria. This includes loss of or replacement of tricar-
boxylic acid (TCA) cycle enzymes and selected subunits of the respiratory chain and induction of peroxiredoxins and defense machinery (4, 8–11). Specific mitochondrial proteases that target oxidatively modified and/or unfolded proteins appear to link protein damage with selective protein degradation (10, 12).

Significant manipulation of mitochondrial functions can also influence oxidative damage elsewhere in the cell, and this can have wide reaching consequences for whole cell/tissue oxidative stress tolerance. For example, overexpression of the mitochondrial Mn-SOD can increase plant stress tolerance (13), and more recently, its knock-out in yeast resulted in the specific oxidation of an array of mitochondrial proteins in the absence of this enzyme (14). Induction of the mitochondrial HSP22 folding chaperones have been reported in response to varying oxidative stresses in plants (15, 16), animals (17), and insects (18). Recently in Drosophila, overexpression of HSP22 has been shown to increase life span and resistance to oxidative stress (19), whereas underexpression decreases life span (20). Complex I of the respiratory chain is a known site of oxidative damage in mitochondria (5), but mutations of complex I can lead to increased stress tolerance and cell viability in plants (21, 22) while often decreasing stress tolerance in animals leading to disease (23, 24). This difference may reflect the presence of alternative NAD(P)H dehydrogenases in plant mitochondria (25). Mitochondria contain an array of ROS protection strategies and antioxidant defense systems to deal with stress-induced damage. Mammalian and yeast mitochondria contain thioredoxin and peroxiredoxin systems (26, 27), whereas plants contain both of these systems (10, 28) as well as an ascorbate/glutathione cycle able to respond to chloroplast-dependent ROS production (29). Animal mitochondria contain uncoupling proteins (UCPs) to lower membrane potential and alleviate high levels of ubiquinone reduction, whereas plants contain both UCPs and non-phosphorylating respiratory bypass proteins allowing non-classical entry and exit of electrons from the respiratory chain (25). Increasingly it will be important to gauge the significance of these oxidative loss-of-function and gain-of-function processes by quantifying their response while accurately mimicking real life stresses that an organism may experience.

We have been investigating this by comparing the reversible chilling and drought treatment of plant leaves with a known irreversible oxidative stress induced by herbicide treatment. Exposure to drought and cold occur widely in cropping and natural ecosystems and leads to increasing ROS production in the chloroplast and to damaged photosynthetic function (30). Microarray analysis suggests significant similarities in the early stages of plant transcriptional response to these two stresses, but the responses then diverge as each stress is prolonged (31, 32). However, the impact of these environments at the protein level generally and for mitochondrial protein and function specifically is much less understood. We have previously shown that modification of lipoic acid on mitochondrial proteins can be observed during all three stress treatments in pea leaves, having direct consequences for the TCA cycle and photorespiratory pathway operations (7). Further investigations of mitochondrial protein import have shown that although both chilling and herbicide treatment debilitated protein import capacity, drought in fact enhanced import capacity, potentially providing a route to protect mitochondria through the accumulation of defense and repair mechanisms during this stress (33). We are now extending these studies, investigating the impact of the stresses on leaf photosynthesis and respiration rates and linking this to changes in protein abundance and protein breakdown in mitochondria.

EXPERIMENTAL PROCEDURES

Materials—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⁻¹ at 24 °C and 65% humidity for 10 days on a 16/8 h day/night cycle.

Stress Treatments—Plants were sprayed evenly with parquat (662.5 mg/liter), allowed to dry, and then returned to controlled environment chambers and exposed to light until harvest. For low temperature treatment, plants were placed at 4 °C for 36 h prior to harvest while maintaining a normal day/night light cycle. For drought treatment, plants were not watered for 7 days prior to harvest while maintaining the same humidity, temperature, and day/night light cycle noted above under “Materials.”

CO₂ Consumption and Synthesis by Intact Leaves—Leaf CO₂ exchange rate was measured using a Licor 6400 gas exchange system (Licor, Lincoln, NE). Gas exchange was measured at targeted leaf chamber irradiances of 1500 μmol m⁻² s⁻¹ photosynthetic photon flux density for the light measurements and in the dark at ambient temperature and humidity. Measurements were initiated by sealing the leaf chamber on a pair of leaves, and once CO₂ and H₂O evolution reached a steady rate (60–90 s) measurements were taken.

TBARS Assay—We used the method of Hodges et al. (34) that takes into account the presence of anthocyanins and sucrose. Assays were performed as noted previously (7).

Pea Mitochondrial Isolation—Mitochondria were isolated according to established methods (35, 36) from ~60 g of 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 KH₂PO₄, 2 mM EDTA, 1 mM glycine, 1% (w/v) polyvinylpyrrolidone-40, 1% (w/v) BSA, 20 mM ascorbate, pH 7.5). The homogenate was filtered through four layers of Miracloth and disrupted with a Polytron (Kinematica, Kriens, Switzerland) in 250 ml of extraction medium containing 0.3 M sucrose, 10 mM TES-KOH, pH 7.5, 5 mM KH₂PO₄, 10 mM NaCl, 2 mM MgSO₄, and 0.1% (w/v) BSA.
Succinate (10 mM), NADH (1 mM), ATP (0.5 mM), and ADP (0.1–1 mM) were added as indicated. Protein concentrations were determined by the method of Peterson (37) using BSA as standard. All mitochondrial preparations were performed independently on three separate sets of treated plants.

2D IEF/SDS-PAGE and BN-PAGE Gels—IEF sample buffer consisted of 6 mM urea, 2 mM thiourea, 2% (w/v) CHAPS, 2% (v/v) ampholytes (pH 3–10), 2 mM tributyolphosphate, and 0.001% (w/v) bromophenol blue. Aliquots of 330 μl were used to reswell dried 180-mm, pH 3–10 non-linear IEF strips (Immobiline DryStrips, Amersham Biosciences) overnight, and then IEF was performed for 19.5 h reaching a total of 49 kV·h at 20 °C on a flat bed electrophoresis unit (Multiphor II, Amersham Biosciences). IEF strips were then transferred to an equilibration buffer consisting of 50 mM Tris-HCl, 15 mM bis-Tris-HCl, 0.02% (w/v) captoethanol and incubated for 20 min at room temperature with rocking. The equilibrated strips were then slotted into central single gel pieces, 10–20 μl of 5% (w/v) Coomassie Blue G250 and 100 mM β-mercaptoethanol and incubated for 20 min at room temperature with rocking. The equilibrated strips were then slotted into central single wells of 4% acrylamide stacking gels above 0.1 × 18.5 × 20-cm, 12% (w/v) acrylamide, 0.1% (w/v) SDS-polyacrylamide gels. Gel electrophoresis was performed at 100 V with circulating cooling (4 °C) and completed in 5 h. Three independent control gels and three independent gels for each treatment were run from different mitochondrial isolations. BN-PAGE was performed largely according to Jansch et al. (38). Gels consisted of a separating gel (5–15% (w/v) acrylamide) and a stacking gel (4% (w/v) acrylamide) formed in a solution of 0.25 M ε-amino-n-capric acid and 25 mM bis-Tris-HCl (pH 7.0). The anode buffer consisted of 50 mM Tricine, 15 mM bis-Tris-HCl, 0.02% (w/v) Coomassie Blue G250 (pH 7.0), and the cathode buffer consisted of 50 mM bis-Tris-HCl (pH 7.0). Mitochondrial sample pellets were suspended to −2 mg/ml in 10 mM TES (pH 7.5), freeze-thawed repeatedly in liquid N₂, and centrifuged for 20 min at 20,000 × g. Membrane pellets were then washed with 10 mM TES (pH 7.5). Aliquots of 1–2 mg of membrane protein were suspended in 75 μl of ACA buffer solution (containing 0.75 M ε-amino-n-capric acid, 0.5 mM NaN₃, EDTA, 50 mM bis-Tris-HCl, pH 7.0) followed by addition of 15 μl of a freshly prepared solution of 10% (w/v) n-dodecylmaltoside. After 10 min of centrifugation at 20,000 × g, 15 μl of 5% (w/v) Coomassie Blue G250 (dissolved in ACA buffer) was added to the supernatants. Gels were run at 4 °C in a precooled apparatus with all samples and buffers precooled to 4 °C. Approximately 30 min before the run, 0.03% (w/v) n-dodecylmaltoside was added to the cathode buffer only. Electrophoresis was commenced at 100-V constant voltage for 45 min and then increased to 15 mA without voltage limitation for 5 h. BN lanes were cut from gels, equilibrated in standard SDS-PAGE sample buffer for 30 min, laid horizontally on 12% acrylamide SDS-PAGE separating gels, and sealed in with 1% agarose, and electrophoreses continued for 5 h under standard conditions. Proteins were visualized by colloidal Coomassie Blue (G250) staining.

Analysis by Progenesis—Quantification of protein abundance following 2D separation of proteins was carried out using the Progenesis version 1.01 (Nonlinear Dynamics, Newcastle upon Tyne, UK) software package. Gels were scanned using a 16-bit charge-coupled device scanner (ProXPRESS, PerkinElmer Life Sciences) using bottom illumination, no excitation, and 680 nm detecting emission. A background exposure of 1–2 s was carried out prior to scanning. Images were scanned at 100-μm resolution and saved as 16-bit tif images. Analysis was carried out using the default analysis wizard, which combines spot detection, warping, and matching on each set of gels (control, drought, chilling, and herbicide). Background subtraction was carried out by “Mode of non-Spot” with a margin of 45. Normalization was carried out by “Total Spot Volume” in which total intensity of pixels of each of the software-delineated polypeptide spots was expressed as a percentage of the total intensity of pixels of all software-delineated polypeptide spots. This normalized the amount of any given polypeptide spot to the total polypeptides on each gel. In this manner 460–580 spots were matched between control and treatment gels. Spots of interest due to changes in abundance were chosen by examining the comparison window in the Progenesis software package; spots that changed greater than 3-fold and spots selected by manual inspection (new spots found in treatments that were not detected as spots in control gel analysis) were then highlighted for further study. Only spots that changed consistently in all three independent sets of gels were finally selected for mass spectrometry identification. These spots were then checked for accurate detection, and where required manual corrections to spot detection were carried out, and spot volumes were redetermined. The relative abundance of each spot per gel was then compared with the control within each of the three treatment groups, and these values were used in the analysis across treatment groups (see Table I).

Q-TOF MS—Selected proteins were selected from the gel, washed, and in-gel digested overnight at 37 °C according to Sweetlove et al. (10). Peptides were extracted from the overnight digests by adding an equal volume of acetonitrile and shaking for 15 min at 8000 rpm on an orbital shaker. The solution was removed from the gel pieces, 10–20 μl of a 50% acetonitrile, 5% formic acid solution was added, and shaking was repeated. The solution was again removed and pooled, and the last step was repeated. Solvent was evaporated from each sample using a SpeedVac (Thermo Savant) for 20–30 min until nearly dried. Samples were hydrated in 16 μl of 5% acetonitrile and 0.1% formic acid prior to mass spectrometric analysis. Samples were analyzed using an Agilent 1100 series capillary LC system and an Applied Biosystems QSTAR Pulsar i LC/MS/MS system equipped with the ion spray source running Analyst QS software (version 1.0 SP8) with the instrument in positive ion mode. Each extracted peptide sample was loaded in turn with the Agilent 1100 series capillary LC system onto a 0.5 × 50-mm C18 reverse phase column (Higgins Analytical) with a C18 OPTI-GUARD guard column (Optimize Technologies) at 16 μl/min equilibrated with 5% acetonitrile and 0.1% formic acid. Peptides were eluted from the C18 reverse phase column into the QSTAR Pulsar i by a 7-min acetonitrile gradient (5–80%) at 16 μl/min under constant formic acid concentrations of 0.1%. During the period of ion detection, eluted peptides were analyzed by the mass spectrometer at 8 μl/min. The total analysis time for each sample was 23 min. The method used to analyze eluted ions uses the information-dependent acquisition capabilities of Analyst QS and the rolling collision energy feature for automated collision energy determination based on the ion m/z (Sciex/AB). The method used a 1-s TOF MS scan that automatically switched (using information-dependent acquisition) to a 2-s product ion scan (MS/MS) when a target ion reached an intensity of greater than 30 counts and its charge state was identified as z = 2+, 3+, or 4+. TOF MS scanning was undertaken on an m/z range of 400–1600 m/z using a Q2 transmission window of 380 amu (100%). Product ion scans were undertaken at m/z ranges of 70–2000 m/z at low resolution utilizing Q2 transmission windows of 50 amu (33%), 190 amu (33%), and 650 amu (34%). Data produced by this method were used for searching the Mascot search engine (Matrix Sciences) for protein identifications. The CID data from each sample were exported from Analyst QS using a purpose built script obtained from Matrix Sciences. The script was set up to centroid the survey scan ions (TOF MS) at a height percentage of 50% and a merge distance of 0.1 amu (for charge state determination), centroid MS/MS data at a height percentage of 50% and a merge distance of 2 amu, reject a CID if less than 10 peaks, and discard ions with charge equal and greater than 5+. Search parameters at Mascot used a peptide tolerance of ±2 Da and MS/MS tolerance of ±1.2 Da, used no variable modifications, and allowed up to one miss cleavage for trypsin digest, and the instrument type was set to ESI-QUAD-TOF.
Searches were performed against the NCBI nr Database (February 11, 2005; 2,321,957 sequences) with a taxonomy restriction to Viridiplantae (green plants) (194,209 sequences).

Supplemental Material—Detailed information of the mass spectra matching, individual peptide scores, and sequence coverage diagrams that form the basis of Table I data are provided in Supplemental Data 1. Generic format data files (Mascot generic) generated from the primary mass spectra based on the script explained above are provided as Supplemental Data 2; these can be directly reanalyzed at www.matrixscience.com by the reader. All 12 of the IEF/SDS-PAGE gels used in the analysis of quantitation shown in Table I are provided in Supplemental Data 3.

RESULTS

Establishing Reversible and Irreversible Plant Stress Regimes—Pea plants were placed under two common and reversible environmental stresses and the terminal oxidative stress of herbicide treatment. For low temperature treatments, plants were chilled at 4 °C for 36 h prior to harvest. For drought treatments, plants were water-restricted for 7 days prior to harvest. After 4–5 days, wilting was evident, and thus ~24–48 h of significant drought stress was imposed by this treatment. For herbicide treatments, plants were treated with paraquat for 12 h prior to harvest. Following all treatments, changes in whole leaf CO2 gas exchange were compared with changes in the properties of isolated mitochondria to understand changes in mitochondria in the context of the observed whole leaf phenotypes. Following stress treatment, chilled and drought-treated plant phenotypes could be fully recovered in the week following removal of the stress, whereas herbicide-treated plants died within a week of treatment (data not shown).

Environmental and Oxidative Stresses Differentially Modified Photosynthetic and Respiratory Rates of Pea Leaves—Infrared CO2 gas analysis was used to measure darkened leaf respiratory rate (Fig. 1A) and illuminated leaf photosynthetic rate (Fig. 1B) of pea leaves following the stress treatments. All three treatments decreased photosynthetic CO2 consumption rate (chilling by 20% and drought by 80%), whereas paraquat abolished CO2 assimilation altogether (Fig. 1B). Dark respiration was also affected by the various treatments but less severely (Fig. 1A). Chilling actually increased respiratory rate 15–20% when measured at 25 °C, indicating that respiratory acclimation to the cold had occurred (39). Drought treatment decreased respiratory rate by 25%, whereas herbicide-treated plants respired in the dark at 20% of control values (Fig. 1A). These stress treatments are often linked to oxidative damage in plants (30), and measuring malondialdehyde (MDA) equivalents on mitochondrial decarboxylating dehydrogenases showed that all treatments dramatically decreased lipoic acid moieties apparent on the H

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"FIG. 1. Effect of environmental stresses on the dark respiration rate, net photosynthetic rate, and accumulation of lipid peroxidation end products in pea leaves. A, rate of carbon dioxide evolution by pea plants in the dark. Values shown are the average of five or more plants per treatment (mean ± S.E.). All three treatments were significantly different from the control (p < 0.05). B, rate of carbon dioxide assimilation by stress-treated pea plants at a light intensity of 1500 μmol m⁻² s⁻¹. Values shown are the average of five or more plants per treatment (mean ± S.E.). All three treatments were significantly different from the control (p < 0.05). C, MDA equivalents measured using the TBARS assay in whole leaf extracts after stress treatments. Values shown are the average of three treatments (n = 3). gFW, gram fresh weight.

whole leaf respiratory rate seen in Fig. 1 could result from damage to the mitochondria themselves or altered substrate availability due to inhibition of photosynthesis. We assessed this by isolating mitochondria from treated leaves and assaying for function and oxidative damage. Maximal electron transport rates with succinate + NADH as substrates were not affected significantly by the treatments (Fig. 2A). However, assessment of lipoic acid moieties on mitochondrial decarboxylating dehydrogenases showed that all treatments dramatically decreased lipoic acid moieties apparent on the H

Mitochondria Isolated from Stressed Pea Leaves Maintain Electron Transport Capacity but Suffer Oxidative Damage and Induce Alternative Respiratory Pathways—The changes in

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protein of glycine decarboxylase (confirming previous results with similar treatments (7)), whereas lipoic acid moieties on pyruvate dehydrogenase complex (PDC) and 2-oxoglutarate dehydrogenase complex were less affected (Fig. 2B). Notably drought treatment appeared to have a differentially greater effect than the other treatments on the lipoic acid moieties attached to both PDC E2 subunits (Fig. 2B). The abundance of alternative oxidase (Aox), a non-phosphorylating respiratory oxidase and a common oxidative stress marker in plant mitochondria, was measured by immunoreactivity (Fig. 2C). In control mitochondria, a single immunoreactive band was evident at 32 kDa (Fig. 2C, lane 1). A new isoform at 31 kDa was induced by drought and herbicide treatment, doubling the overall abundance of Aox protein, but no significant change was recorded in the chilling treatment (Fig. 2C). Antibodies to the plant uncoupling protein specifically identified two protein bands in pea mitochondria; the 30-kDa isoform doubled in abundance during drought treatment, but neither isoform was induced by the other stress treatments (Fig. 2D). The induction of Aox and UCP and the greater modification of lipoic acid (Fig. 2B) during drought treatment indicate that this is a more potent oxidative stress in pea mitochondria than the chilling treatment.

**Fig. 2.** Effect of environmental stresses on lipoic acid moieties of GDC, Aox, and NADH/succinate-dependent respiration in isolated pea leaf mitochondria. **A**, rate of oxygen consumption by mitochondria isolated from the leaves of stress-treated pea plants with NADH + succinate as substrates. Values shown are the state 3 rates in the presence of ADP and are the average of three treatments (mean ± S.E.). All three treatments were not significantly different from the control (p > 0.3). **B–D**, mitochondria were isolated from stress-treated pea plants, and their proteins were separated by SDS-PAGE. Numbers on the left represent apparent molecular masses in kDa, and numbers below the blots are the percentage intensity of the band relative to the control. **B**, the Lip A panel (17 kDa) shows the H protein of GDC and the E2 subunits of PDC and 2-oxoglutarate dehydrogenase complex (OGDC) detected using an anti-lipoic acid antibody. **C**, the Aox panel shows Aox proteins detected with an anti-Aox antibody. **D**, the UCP panel shows the UCP proteins detected with an anti-UCP antibody.
in abundance of proteins was measured in control gels compared with each treatment in three independent replicates using the Progenesis (Nonlinear Dynamics) software package. Variable amounts of a string of protein spots marked with an asterisk (*) in Fig. 3 were not included in the analysis as these were identified as being derived from bovine serum albumin used in the wash medium for mitochondrial isolation. A set of 33 protein spots were identified that appeared to change significantly in abundance across the replicated sets in at least one stress treatment compared with control gels (see “Experimental Procedures” for details of selection). Each of these spots of interest was extracted from the gel and in-gel digested with trypsin, and tandem mass spectrometry was performed on the resulting peptides yielding MS/MS spectra to aid protein identification. The results of these analyses are summarized in Table I. All but one of the 33 proteins could be identified by the mass spectrometry approach. Of these, 29 are likely to be of mitochondrial origin based on identifications and comparison to known mitochondrial proteins from model plants (40–42). The β-carbonic anhydrase (Spot 27) and Rubisco small subunit (Spot 32) and a breakdown product of the large subunit (Spot 31) were clearly chloroplast contaminants. The Cu/Zn-SOD (Spot 33) is classed as a cytosolic protein but also occurs in the mitochondrial intermembrane space in plants and yeast (43). Among the likely mitochondrial proteins matched to proteins from a number of other species (Table I), six increased in abundance, five decreased in abundance, and 18 increased in abundance but were likely breakdown products based on the gel-based size of the identified protein spots. Sometimes the breakdown products mirrored decreases in specific proteins. For example, decreases in the P protein of glycine decarboxylase (GDC) (Spot 4) were mirrored by an increase in a 31-kDa breakdown product of P protein matched to the same gene (Spot 5). Similarly decreases in T protein of GDC (Spots 6 and 7) were mirrored by an increase in a 28-kDa breakdown product matching the same gene (Spot 8). Decreasing abundance of specific parent proteins and the increase of their breakdown products are listed consecutively in Table I for simplicity of presentation. Furthermore the proteins identified are listed in their major function classes: GDC and serine hydroxymethyltransferase (SHMT) subunits, TCA cycle enzymes, oxidative phosphorylation complex subunits, heat shock proteins, and a diverse set of miscellaneous proteins (Table I).

The H, P, T, and L subunits of GDC were clearly damaged during stress treatments. This was most dramatic in paraquat treatments but was also evident in the environmental stresses. This damage was more evident in chilling than in drought treatments (note especially Spots 4, 5, 6, 8, 9, and 10) despite chilling seeming to be the milder stress based on the data presented in Figs. 1 and 2. Decreases in abundance of over 80% for P, T, and SHMT proteins were recorded after paraquat treatment (Spots 4, 6, 7, and 9). During drought these decreases were 0–30%, whereas 20–60% decreases were recorded following chilling. Interestingly H protein did not seem to be significantly degraded during stresses (Spots 2 and 3), but as shown earlier, the lipoic acid group on this protein was extensively damaged during all three stress treatments (Fig. 2B).

Breakdown products for malate dehydrogenase (Spot 13) and PDC E1β (Spot 15) were recorded in paraquat treatments but was also evident in the environmental stresses. This damage was more evident in chilling than in drought treatments (note especially Spots 4, 5, 6, 8, 9, and 10) despite chilling seeming to be the milder stress based on the data presented in Figs. 1 and 2. Decreases in abundance of over 80% for P, T, and SHMT proteins were recorded after paraquat treatment (Spots 4, 6, 7, and 9). During drought these decreases were 0–30%, whereas 20–60% decreases were recorded following chilling. Interestingly H protein did not seem to be significantly degraded during stresses (Spots 2 and 3), but as shown earlier, the lipoic acid group on this protein was extensively damaged during all three stress treatments (Fig. 2B).
### TABLE I
Identification of IEF/SDS-PAGE-separated proteins spots from isolated pea mitochondria from mock (M)-treated, chilled (C), drought (D)-treated, and paraquat (P)-treated plants

Spot, spot number corresponding to spots in Fig. 4; BD, designated breakdown product based on comparison of expected and observed molecular mass indicated by *; Change, the average change in abundance from three independent treatments (± S.D.); Protein ID, matched protein description; Accession, accession number from NCBI Database of Matched protein; Species, the species of the matched protein; Mr, Gel (kDa), apparent molecular mass of protein on gel; Mr Pre (kDa), predicted molecular mass based on amino acid sequence; MOWSE, score obtained from Mascot for each match; Coverage, percentage of the protein match; NP, the number of matched peptides; DH, dehydrogenase.

| Spot | BD | Change | M | C | D | P | Protein ID | Accession | Species | Mr Gel (kDa) | Mr Pre (kDa) | MOWSE | Coverage (%) | NP |
|------|----|--------|---|---|---|---|-----------|-----------|---------|-------------|-------------|--------|--------------|----|
| 1    | *  |        | 29 | 53|    |    | GDC L-protein/dihydrolipoamide DH | gi100037  | Pisum sativum | 726         | 32          | 18     |
| 2    |    |        | 18 | 14|    |    | GDC H-protein                  | gi999900  | Pisum sativum | 286         | 60          | 7      |
| 3    |    |        | 16 | 18|    |    | GDC H-protein                  | gi9955326 | Pisum sativum | 273         | 70          | 11     |
| 4    |    |        | 110| 115|    |    | GDC P-protein                  | gi20741   | Pisum sativum | 973         | 24          | 28     |
| 5    | *  |        | 31 | 115|    |    | GDC P-protein                  | gi20741   | Pisum sativum | 369         | 9           | 12     |
| 6    |    |        | 44 | 44|    |    | GDC T-protein                  | gi3021553 | Pisum sativum | 849         | 48          | 29     |
| 7    |    |        | 42 | 44|    |    | GDC T-protein                  | S55661    | Pisum sativum | 791         | 44          | 25     |
| 8    | *  |        | 28 | 44|    |    | GDC T-protein                  | gi3021553 | Pisum sativum | 571         | 31          | 19     |
| 9    |    |        | 55 | 57|    |    | SHMT                          | gi282928  | Pisum sativum | 889         | 60          | 28     |
| 10   | *  |        | 27 | 57|    |    | SHMT                          | gi282928  | Pisum sativum | 239         | 23          | 9      |
| 11   | *  |        | 29 | 57|    |    | SHMT                          | gi282928  | Pisum sativum | 480         | 31          | 13     |
| 12   |    |        | 22 | 36|    |    | NAD-dependent malate DH        | gi2827080 | Medicago sativa | 300         | 24          | 8      |
| 13   | *  |        | 28 | 39|    |    | PDC Eiβ                        | gi1336097 | Pisum sativum | 93          | 6           | 3      |
| 14   | *  |        | 42 | 55|    |    | ATPase α                       | gi100086  | Pisum sativum | 474         | 27          | 14     |
| 15   | *  |        | 31 | 55|    |    | ATPase α                       | gi100086  | Pisum sativum | 626         | 32          | 19     |
| 16   | *  |        | 31 | 55|    |    | ATPase α                       | gi100086  | Pisum sativum | 743         | 30          | 21     |
| 17   | *  |        | 42 | 60|    |    | ATPase β                       | gi7436097 | Pisum sativum | 224         | 15          | 9      |
| 18   | *  |        | 32 | 36|    |    | ATPase γ                       | gi303626  | Ipomoea batatas | 159         | 13          | 5      |
| 19   | *  |        | 31 | 41|    |    | ATPase γ                       | gi31431085 | Oryza sativa | 149         | 8           | 4      |
| 20   |    |        | 80 | 81|    |    | Complex I 75 kDa subunit       | At5g37510 | Arabidopsis thaliana | 230         | 10          | 9      |
paraquat treatments, but chilling yielded higher abundances of breakdown products than did drought treatment. There was no evidence that the intact subunits for the ATP synthase were decreasing in abundance, suggesting that the observed damage was only a small percentage of the total and not likely to have a large impact on total ATP synthase activity. Changes in the abundance of two subunits of complex I were observed: the 75-kDa subunit decreased during stress, whereas the B13 subunit appeared to increase in drought and herbicide treatments (Spots 20 and 21).

Marked increases in abundance were recorded in the heat shock proteins HSP90, HSP70, and HSP22. The HSP22 increases were consistently found following all three stress treatments, whereas the HSP70 and HSP90 abundance increases only occurred in chilling and drought treatments (Spots 20 and 21).

Marked increases in abundance were recorded in the heat shock proteins HSP90, HSP70, and HSP22. The HSP22 increases were consistently found following all three stress treatments, whereas the HSP70 and HSP90 abundance increases only occurred in chilling and drought treatments. Interestingly HSP70 appeared to decrease in abundance in herbicide treatment (Spot 23), consistent with the formation of a 10-kDa smaller protein matching to the same gene product (Spot 24).

Among the miscellaneous protein set, NDPK3 abundance decreased markedly following herbicide treatment but was much less affected by chilling and unaffected by drought (Spot 30). Chaperonin-10 increased with stress severity, whereas the likely intermembrane space-localized Cu/Zn-SOD protein increased but only in drought treatment (Spot 33). A breakdown product for the mitochondrial processing peptidase (MPP, Spot 29) was observed following herbicide stress. The intact chloroplast contaminants (Spots 27 and 32) decreased during all stresses, whereas the breakdown product of the chloroplastic Rubisco large subunit increased in chilling and herbicide treatments (Spot 31).

**Abundances of Intact Oxidative Phosphorylation Complexes Were Not Significantly Affected by Oxidative and Environmental Stresses**—To directly consider the impact of the stress treatments on the integrity of the respiratory chain and F$_{1}$F$_{0}$ ATP synthase complexes, mitochondrial membrane protein samples were separated by blue native-PAGE. The resulting first dimension gels were then run on SDS-PAGE slab gels, producing two-dimensional BN/SDS-PAGE gels, which were stained with colloidal Coomassie Blue (Fig. 4). Comparing these gels with previously reported separations of plant mitochondrial membrane proteins revealed the presence of...
We have compared a chemical stress (paraquat) with the more physiological stresses of drought and cold and examined their impact on leaf mitochondrial proteins. The drought and chilling regimes we used did not cause accumulation of lipid peroxidation products (MDA equivalents) significantly above controls (Fig. 1C), but these treatments clearly affected leaf metabolism and caused oxidative modification of mitochondrial proteins (Figs. 2 and 3). Presumably the oxidative stress imposed by these environmental treatments is more localized than the more severe oxidative stress resulting from the interaction of paraquat with the photosynthetic machinery that causes extensive production of H$_2$O$_2$.

Significant similarities exist between the mitochondrial proteins observed to decrease in abundance here (Table I) and those observed to change in the study of chemical oxidative stress of mitochondria from Arabidopsis suspension cells (10) and the proteins shown to be modified by in vitro oxidation in rice mitochondria (3). Notably matrix carbon metabolism enzymes appear to be major targets for oxidative modification and breakdown in vitro and in vivo. Less degradation of F$_1$F$_0$ ATP synthase subunits occurs, whereas the electron transport chain complexes are the least affected. Interestingly NDPK3, a mitochondrial intermembrane space protein known to be involved in response to heat stress (46), and the 75-kDa subunit of complex I were observed to decrease in both Arabidopsis cell suspension mitochondria (10) and pea leaf mitochondria on IEF/SDS-PAGE gels (Fig. 3). We provide clear evidence that the multiple glycine decarboxylase subunits and SHMT are highly susceptible to damage and breakdown during environmental stresses in plants. Kristensen et al. (3) noted GDC and SHMT as sites of carbonyl formation following an in vivo oxidation protocol in rice, whereas Johansson et al. (2) identified these same proteins as the major oxidized proteins in mitochondria of Arabidopsis. However, the susceptibility of GDC and SHMT to breakdown was much lower in drought-stressed leaves than in chilled leaves despite clear evidence that drought-stressed leaves were more oxidatively stressed (Fig. 2) and had slower rates of photosynthesis and respiration (Fig. 1).

We have previously reported that following these stress treatments, protein import into isolated pea mitochondria was differentially modulated (33). Notably drought caused a specific increase in import rate via the general import pathway, whereas herbicide and chilling stress led to inhibition of both the general and carrier import pathways. Using antibodies raised against HSP70 and MPP, we observed no increase upon stress treatment but a 30% decrease in MMP following herbicide treatment (33). Here we observed the marked increase in an MMP subunit breakdown product specifically following herbicide treatment (Table I, Spot 29), which is consistent with this previous observation. Interestingly we also observed an increase in a specific HSP70 protein (Table I,
Spot 23) in both chilled and drought-treated leaves. This increase of a specific isoform might help explain the observation of general import pathway-specific induction by stress (33) as HSP70 is both a folding chaperone and the ATP-dependent motor for protein import via the general import pathway but notably is not required for carrier import into the inner membrane (47).

One of the main contrasting results observed here was clear induction of HSPs from pea leaves following environmental stress, especially HSP22s, whereas no such induction was observed in chemically stressed Arabidopsis cell suspension cultures (10). This induction is consistent with a series of reports highlighting HSP22 mRNA and protein induction during environmental stress in plants (15, 48) and the linkage of HSP22 induction with cross-tolerance of plants to other oxidative stresses (16). The signal for mitochondrial HSP upregulation has been investigated recently in maize plants and shown to likely originate from decreased mitochondrial membrane potential (22) rather than an oxidative pathway per se, suggesting that onset of respiratory deficiency is a key element in HSP22 and HSP70 induction. As respiratory electron transport chains were not greatly affected during our stresses (Figs. 2 and 4), it is likely that decreased internal NADH production due to losses in GDC and TCA cycle enzymes (Table I and Fig. 3) is the main signal for HSP induction during these stresses.

Oxidative stress is known to lead to cell death in both animals and plants by either a necrotic route after substantial cell damage or via an ordered and controlled pathway termed programmed cell death (PCD). Mitochondria play a key role in PCD in animals where release of cytochrome c and/or other intermembrane space proteins is a step of commitment to the PCD route (49). In plants, programmed cell death can also occur via mitochondria-dependent pathways. Cytochrome c release is known in plant PCD (50), and a range of mitochondrial protein changes have been associated with PCD in plants (51), but the mechanisms of PCD in plants and its regulation are still an issue of current research (52–54). It is also clear from this latter literature that PCD is not a reversible process in plants or animals following mitochondrial membrane permeability transitions and cytochrome c release. In our study, chilling and drought were reversible and did not lead to large scale cell death in pea leaves. Herbicide treatment was not reversible, but given the intensity of the oxidative assault it is likely that necrosis rather than PCD occurs eventually in these leaves. Furthermore none of the proteins highlighted to be associated with PCD in plant mitochondria by Swidzinski et al. (51) were identified in our study of protein expression differences (Table I). Therefore, it is most likely that our study is showing stress responses in mitochondria rather than providing a window into programmed cell death pathways.

A common theme, however, does emerge when comparing our results with those obtained in mitochondria isolated from a variety of other organisms under oxidative stress. Although the proteins of the electron transport chain appear to be oxidatively modified in both plants and animals (3–5), respiratory complex function is often relatively resistant to oxidants and oxidative modification. However, matrix-facing inner membrane proteins are damaged very readily (4), and the soluble matrix contains an array of carbon metabolism proteins that are functionally damaged and readily degraded following modification in mammals (9, 55), yeast (14), and plants (Ref. 10; Table I and Fig. 3). A range of defense strategies appears to exist that may help to minimize this damage: 1) induction of defenses to lower ROS production from the electron transport chain (UCP in both mammals and plants and Aox in plants); 2) induction or mobilization of direct antioxidant defenses to aid decrease in mitochondrial ROS production and protect soluble enzymes; 3) protection of existing matrix enzymes by synthesis of soluble protein-folding molecular chaperones like HSP22, HSP70, and HSP90; and 4) enhancement of import characteristics to maximize protein replacement as shown in plants (33) and implied in the response of human mitochondria to hydroperoxide stress (11). However, the level of damage and the induction of defense responses do not appear to operate equally under all stress conditions. Their implementation will depend on the timing, length, and reversibility of the stress conditions and may include both ROS-dependent pathways of stress response and -independent pathways associated with cellular detection of respiratory deficiency. Here drought stress induced more defense strategies and showed less protein damage than chilling, whereas the terminal oxidative stress of paraquat showed only selective defense protein accumulation in mitochondria while causing oxidative damage and degradation of large numbers of important enzymes. Comparing the level and timing of induction of stress response/defense proteins with the degree of protection across a range of stress treatments will help identify new putative causative agents in mitochondrial stress tolerance in plants.

Acknowledgment—Dr. Erik Veneklaas (University of Western Australia, Perth, Australia) is thanked for helpful discussions on gas exchange measurements.
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