Oxygen Initiation of Respiration and Mitochondrial Biogenesis in Rice

Katharine A. Howell, Kim Cheng, Monika W. Murcha, Linne E. Jenkin, A. Harvey Millar, and James Whelan

From the Australian Research Council Centre of Excellence in Plant Energy Biology, University of Western Australia, Perth, Western Australia 6009, Australia

Rice growth under aerobic and anaerobic conditions allowed aspects of mitochondrial biogenesis to be identified as dependent on or independent of an oxygen signal. Analysis of transcripts encoding mitochondrial components found that a subset of these genes respond to oxygen (defined as aerobic), whereas others are relatively unaffected by oxygen availability. Mitochondria formed during growth in anaerobic conditions had reduced protein levels of tricarboxylic acid cycle components and cytochrome-containing complexes of the respiratory chain and repressed respiratory functionality. In general, the capacity of the general import pathway was found to be significantly lower in mitochondria isolated from tissue grown under anaerobic conditions, whereas the carrier import pathway capacity was not affected by changes in oxygen availability. Transcript levels of genes encoding components of the protein import apparatus were generally not affected by the absence of oxygen, and their protein abundance was severalfold higher in mitochondria isolated from anaerobically grown tissue. However, both transcript and protein abundances of the subunits of the mitochondrial processing peptidase, which in plants is integrated into the cytochrome bc1 complex, were repressed under anaerobic conditions. Therefore, in this system, an increase in import capacity is correlated with an increase in the abundance of the cytochrome bc1 complex, which is ultimately dependent on the presence of oxygen, providing a link between the respiratory chain and protein import apparatus.

Motivation

Mitochondria are best known for their role in respiration and ATP production via oxidative phosphorylation, a process that inherently requires the presence of oxygen. Detailed molecular studies have begun to show that changes in oxygen levels are incorporated into transcriptional and post-transcriptional mechanisms in cells and have a profound effect on mitochondrial function and biogenesis. Most of these studies examining eukaryotic responses to oxygen deficit have been performed using the facultative anaerobe, Saccharomyces cerevisiae (yeast), where growth under aerobic and anaerobic conditions is associated with changes in mitochondrial ultrastructure and respiratory activity (1, 2). Studies using the yeast system and examining oxygen-regulated gene expression have resulted in genes being classified based on their transcriptional response to oxygen levels. Hypoxic genes are transcriptionally up-regulated under anaerobic conditions, whereas aerobic genes are transcribed optimally under aerobic conditions (3). Interestingly, many of the aerobic genes identified and examined in yeast encode mitochondrial electron transport chain components, whereas other mitochondrial components, such as subunits of the protein import apparatus, are not oxygen-responsive at the transcription level (4–6). Considerable progress has been made in understanding the regulation of these hypoxic and aerobic genes, revealing a complex interaction between transcription factors that are ultimately dependent on feedback regulation by heme, whose synthesis in the cell is dependent on the availability of molecular oxygen (7). Studies in yeast have also focused on the expression of subunits of the heme-containing cytochrome c oxidase complex, and a role for this enzyme in oxygen sensing has been proposed (8).

In contrast to the situation in yeast, an understanding of how multicellular organisms respond to anaerobic conditions is limited. Many eukaryotes require oxygen for survival; therefore, studies investigating the effect of oxygen deprivation are challenging, since the viability of the organism of interest is usually compromised by the experimental conditions. However, some plants show substantial tolerance to anaerobic conditions. The most well known is rice, which is able to germinate and grow for days in the absence of oxygen (9–11). Historically, research into the effects of oxygen deprivation on mitochondria in plants has focused on ultrastructural changes to the appearance of these organelles and have shown that mitochondrial ultrastructure is maintained in anoxia-tolerant plant species, whereas a breakdown of the mitochondrial membrane structure is seen in cells of anoxia-intolerant species (12). Studies investigating the effect of oxygen deficit on mitochondrial function, in relatively mature rice tissues, have focused on changes in the abundance and activity of respiratory chain complexes and tricarboxylic acid cycle enzymes (11, 13–16). Notably, a lack of heme-containing respiratory chain components in mitochondria isolated from tissue grown under anaerobic conditions and a rapid production of these components during reaeration has been shown (16), which appears to be analogous to the heme-linked oxygen response investigated in yeast and animals. However, although...
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these studies show the final result of anaerobic or aerobic growth in rice, they have not specifically probed the nature of the oxygen-dependent events in transcriptional activation and mitochondrial biogenesis that produce these outcomes in plants. Further, specific differences exist between yeast and plants in terms of the heme synthesis pathway and the composition of the mitochondrial heme-containing respiratory complexes. First, the reactions culminating in heme synthesis occur in plastids and the cytosol in plants linking both chlorophyll and heme production (17). Second, the mitochondrial processing peptidase, which is essential for removal of presequences from imported proteins, is incorporated into the heme-containing cytochrome bc1 complex of the respiratory chain in plants (18–21), whereas in yeast and animals, the processing peptidase is a soluble complex in the mitochondrial matrix (22–24).

We have previously established a system for examining mitochondrial biogenesis during rice germination and early seedling development under normal growth conditions (25), where we have found that the maturation of mitochondria is facilitated by high levels of protein import components already present in promitochondria within the dry seed and which is driven by oxidation of external NADH allowing for the rapid resumption of respiratory and metabolic functions to support early seedling establishment. In this study, we have examined the difference between rice germination and early growth under anaerobic and aerobic conditions to understand the transcriptional oxygen response of genes for plant mitochondrial proteins. We have followed the impact of these changes on mitochondrial biogenesis through a series of events leading from the differential transcriptional response, to altered operation of the general and carrier protein import apparatus, to the selective accumulation of the mitochondrial proteome.

EXPERIMENTAL PROCEDURES

Plant Material—Dehulled, sterilized rice seeds (Oryza sativa cv. Amaroo) were germinated in the dark, submerged in rice growth medium (0.5 mM MES,3 0.5 mM CaCl2, pH 6.5) with carbencillin (6 mg/liter), and incubated at 30 °C. Flasks were continuously bubbled with air or nitrogen, to generate aerobic or anaerobic conditions, respectively, using spargers below the water level. For electron microscopy, mitochondrial isolation, and nucleic acid isolation, rice embryos were manually dissected from grains.

Electron Microscopy—Rice embryos were fixed in 2.5% (v/v) glutaraldehyde in 0.05 m phosphate buffer (pH 7.0) for 24 h, postfixed with 1% (w/v) osmium tetroxide, dehydrated in an ethanol series and embedded in Spurr’s resin. Thin sections (60–100 nm) of shoot apex tissue were prepared using glass knives and a semiautomatic ultramicrotome (Reichert-Jung, Vienna, Austria), collected on copper grids, poststained with uranyl acetate (1% (w/v) and lead citrate (26) and examined using a JEOL 2000FX-II transmission electron microscope (Tokyo, Japan).

Mitochondrial Isolation—Mitochondria were isolated from rice embryos using a modified mitochondrial isolation protocol, as described previously (25). Yields of mitochondrial protein were determined using the Coomassie Plus protein assay reagent according to the manufacturer’s instructions (Pierce).

Respiratory Measurements—For respiratory measurements on isolated mitochondria, 80–200 μg of mitochondrial protein was added to 600 μl of reaction medium (0.3 m mannitol, 10 mM TES, 5 mM KH2PO4, 10 mM NaCl, 2 mM MgSO4, 0.1% (w/v) bovine serum albumin, pH 7.5), and oxygen consumption was measured at 25 °C in a Clarke-type oxygen electrode. The following reagents and inhibitors were added to the reaction medium to examine mitochondrial function: ATP (0.3 mM), succinate (5 mM), ADP (0.25 mM), NADH (1.5 mM), myxothiazol (5 μM), nigericin (10 μM), cytochrome c (50 μM), Triton X-100 (0.05%, w/v), and KCN (1 mM).

Western Blotting and Immunodetection—Mitochondrial protein samples were separated by SDS-PAGE (10 μg/lane), transferred to a nitrocellulose membrane, and analyzed using antibodies raised to mitochondrial proteins. The F1α subunit of ATP synthase, HSP70, the E1α subunit of the pyruvate dehydrogenase complex (PDH), and the outer mitochondrial membrane voltage-dependent anion channel were identified with monoclonal antibodies raised to maize mitochondrial proteins (Dr. Tom Elthon, University of Nebraska, Lincoln, NE). Lipoic acid attached to the acyltransferases (E2) in 2-oxoglutarate and pyruvate dehydrogenase complexes was detected with a polyclonal antibody raised against lipoic acid (27). Antibodies raised against cytochrome c, Cox2 (cytochrome oxidase subunit 2) and HSP60 were purchased from BD Biosciences PharMingen, Agrisera (Stockholm, Sweden) and StressGen (Victoria, Canada), respectively. The Rieske iron-sulfur protein (RISP) was detected using an anti-tobacco RISP antibody (Dr. Dean Price, Australian National University, Canberra, Australia). TOM20 (translocase of the outer membrane subunit of 20 kDa) was detected using an antibody raised against the Arabidopsis TOM20-3 isoform (28). Immunoreaction was detected using the BM luminescence Western blotting kit (Roche Applied Science) and visualized using a LAS 1000 (Fuji, Tokyo, Japan).

Cloning and Generation of Quantitative PCR Standards—For each gene of interest, a complete or partial fragment was amplified from rice cDNA or genomic DNA using the Expand High Fidelity PCR system (Roche Applied Science) and cloned into the pCR2.1®-TOPO® vector using the TOPO TA cloning® kit (Invitrogen). Primers used to clone genes have been previously described (25) and for MPPα (mitochondrial processing peptidase) (Os01g09560), MPPβ (Os03g11410), and Adh1 (alcohol dehydrogenase) (Os11g10480) included the following (5′–3′): MPPα-FWD, CTTGATTGGGAAGTGTAAGTCAGG; MPPα-REV, TCATTTCGAGCGAAACTTG; MPPβ-FWD, TCATGTGCCAAGACTTGC; MPPβ-REV, CTAGTAACGGGAGCATGTAGG; Adh1-FWD, ATGGCGACAGCGGGAAAGG; Adh1-REV, CCAAGCAGATACACACATTC. Linear standards for real-time PCR were prepared as previously described (29).

3 The abbreviations used are: MES, 4-morpholineethanesulfonic acid; HSP, heat shock protein; LEA, late embryogenesis abundant; PDH, pyruvate dehydrogenase complex; Pc, phosphate carrier; RISP, Rieske iron-sulfur protein; SOM, self-organizing map; TES, 2-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino)ethanesulfonic acid; MS, mass spectrometry.
RESULTS

Mitochondrial Ultrastructure Is Independent of Oxygen Availability but Rice Embryo Growth and Respiratory Activity Are Compromised under Anaerobic Conditions—To ensure our experimental protocols were replicating the aerobic and anaerobic growth of rice reported by other workers, we initially undertook a broad survey of rice germination physiology and respiratory function. Dehulled rice grains were grown for 48 h submerged in growth media continuously bubbled with air or commercial grade nitrogen to create aerobic (A) or anaerobic (N) conditions, respectively. Although germination occurred under both conditions, differences in embryo morphology were found (Fig. 1A, top). Coleoptile growth was slower in anaerobically grown rice, and most obviously, the radicle did not elongate, consistent with the requirement for trace amounts of oxygen for root development (34). Rice grains were also germinated and grown in anaerobic conditions for 24 h, followed by a switch to growth in aerobic conditions for 48 h or germinated and grown in aerobic conditions for 24 h, followed by a switch to growth in anaerobic conditions for 48 h (Fig. 1A, bottom). Using this switch system, differences in morphology were also observed as the embryos developed. As observed previously, rice germinated in the presence of oxygen initially showed slightly faster coleoptile growth compared with rice grown without oxygen (see the 24 h time point). However, after switching the conditions and allowing growth for a further 48 h (i.e. at the 72 h time point), clear differences were evident, with grains switched to an anaerobic environment showing no root development, whereas extensive root elongation was observed in those grains that were switched into aerobic conditions, indicating that the introduction or removal of oxygen after germination can also alter development.

Transmission electron microscopy was used to assess changes in mitochondrial morphology in rice embryo tissue after growth in aerobic and anaerobic conditions (Fig. 1B). The transition from promitochondria, showing little discernible internal structure (25), in the dry seed (0 h) to typical mature mitochondria, characterized by an increase in

Nucleic Acid Isolation and cDNA Preparation—Total RNA was isolated from rice embryos using the RNeasy® Plant Mini Protocol (Qiagen, Clifton Hill, Australia) in combination with the RNase-free DNase Set (Qiagen) and the DNA-free™ kit (Ambion, Austin, TX). Three independent RNA preparations were performed for each developmental stage/growth condition, and the concentration of RNA was determined spectrophotometrically. cDNA was prepared from 1 μg of total RNA, in duplicate, using random primers (p(dN)₆, 100 pmol; Roche Applied Science) and Expand Reverse Transcriptase (Roche Applied Science). cDNA samples were purified using the QIAquick® PCR purification kit (Qiagen) and diluted 1:10 with water and with a final concentration of 0.008% (w/v) bovine serum albumin.

Quantitative PCR Analysis—Quantitative PCR was performed using the iCycler instrument with iQ™ SYBR® Green Supermix (Bio-Rad), using 25-μl reaction volumes, under conditions optimized to minimize primer-dimer formation and maximize amplification efficiency. To determine changes in transcript abundance, cDNA generated from total RNA isolated from rice embryos was used as a template. For SOM analysis of transcript profiles using GeneCluster 2.0 (version 2.1.7; available on the World Wide Web at www.broad.mit.edu/cancer/software/genecluster2/gc2.html) (59) default settings for both basic and advanced parameters were used. Sequences of all primers used for quantitative PCR have been previously described (25) and for MPPα, MPPβ, and Adh1 included the following (5’–3’): Q-MPPα-FWD, GAAACTGAAGGCAGA- MPPβ-FWD, GTTATTCTGCGAGAGATGG; Q-MPPβ-REV, GGAGCAGTGTAATTCTCAGC; Q-Adh1-FWD, GTTGC TTCTTATGCTCAGG; Q-Adh1-REV, CAAATCTGTGCGGTTCCAGG.

Two-dimensional Gel Separation—Rice embryo mitochondrial samples (250 μg) were acetone precipitated and isoelectric focusing/SDS-PAGE analysis was carried out as previously described (30) using nonlinear pH 3–10 isoelectric focusing strips. Gels were stained using colloidal Coomassie (17% (w/v) ammonium sulfate, 34% (v/v) methanol, 3% (v/v) phosphoric acid, 0.1% (w/v) Coomassie Brilliant Blue G250) and destained using 0.5% (v/v) phosphoric acid to visualize protein spots. Gels were scanned using a 12-bit transparency scanner (Image Scanner; Amersham Biosciences). Protein spots were excised and prepared for mass spectrometry as previously described (30). Changes in spot intensity were analyzed by ImageMaster two-dimensional Elite software (Amersham Biosciences) on 12-bit TIFF images with background subtraction.

Mass Spectrometry—Proteins of interest were analyzed by electrospray ionization-tandem mass spectrometry (MS/MS) using a QStar Pulsar MS/MS system (Applied Biosystems, Foster City, CA). Primary MS/MS data were analyzed using the Mascot server (available on the World Wide Web at www.matrixscience.com/), searching against an in house data base comprising TIGR and NCBI rice protein sets with error tolerances of MS of ±1.2 Da and MS/MS of ±0.6 Da.

In Vitro Import Assays—Precursor proteins were generated from the soybean alternative oxidase (31), soybean F₄₅₆ subunit of ATP synthase (32), maize phosphate carrier (obtained from Prof. C. Leaver, University of Oxford, Oxford, UK), Arabidopsis Tim23 (translocase of the inner membrane 23) (29), Arabidopsis Rps10, and lettuce (Lactuca sativa) Rps10 cDNA clones using the TN-T coupled reticulocyte lysate system (Promega, Melbourne, Australia) in the presence of [³⁵S]methionine. Imports were performed with 20 μg of mitochondrial protein as previously described (33), and substrates were included to final concentrations as follows: ATP (0.75 mM), NADH (10 mM), and succinate (5 mM). Precursor proteins were added, and the import reaction was left to incubate for 10–60 min at 26 °C. For precursors with a cleavable presequence, this was followed by treatment by proteinase K, whereas for the unprocessed precursors, proteinase K treatment followed preparation of mitoplasts via osmotic swelling (29). Mitochondria or mitoplasts were then pelleted by centrifugation of the import reaction at 20,800 × g at 4 °C for 2 min. Mitochondrial proteins were separated by SDS-PAGE, and gels were stained, dried, and exposed to a BAS TR2040S plate for 48 h. Radiolabeled proteins were detected using a BAS 2500 (Fuji).
matrix density and formation of cristae, occurred in both the presence and absence of oxygen.

To determine mitochondrial functionality under the different growth conditions indicated (Fig. 1A), the respiratory activity of isolated rice embryo mitochondria was determined (Fig. 1C). Succinate-dependent respiration, succinate plus NADH-dependent respiration, and cytochrome c oxidase activity were measured. For mitochondria isolated from tissue grown under aerobic conditions, oxygen uptake in the presence of succinate or succinate plus NADH showed a steady increase over the 48-h time period. Cytochrome c oxidase activity also increased but only after 24 and 48 h postimbibition (p < 0.05, n = 3), with no significant difference in activity determined between mitochondria isolated from dry seed (D) and after 12 h of growth under aerobic conditions (12A). For anaerobically grown tissue, mitochondrial respiration (dependent on succinate or NADH plus succinate) as well as cytochrome c oxidase activity showed no significant differences over the 48-h growth period (p < 0.05, n = 3). Using the switch system, mitochondria isolated from tissue grown under aerobic conditions followed by anaerobic conditions (24A/24N) showed that respiratory activity driven by succinate as well as cytochrome c oxidase activity was maintained at 24A levels, indicating that increases to 48A levels were repressed by transfer to anaerobic conditions. Furthermore, respiration driven by succinate plus NADH decreased from 24A levels. Switching from anaerobic to aerobic conditions (24N/24A) resulted in increases in all oxygen consumption measurements compared with 24N levels, reaching levels equal to or exceeding those seen for 24A mitochondria.

Transcript Levels of Mitochondrial Components Are Differentially Affected by Oxygen Availability—Transcript levels of 28 genes encoding mitochondrial proteins were then evaluated in rice embryo tissue under continuous growth in either aerobic (shown in red) or anaerobic (shown in blue) conditions using...
quantitative reverse transcription-PCR (Fig. 2). These genes encoded components of the mitochondrial import apparatus, tricarboxylic acid cycle, electron transport chain, and mitochondrial transcription and translation machinery. Transcript profiles were grouped using SOM clustering to determine if the changes in transcript levels differed between growth conditions (Fig. 2A). Interestingly, although some showed a large response to changes in oxygen levels (Group 1), others were not significant.

**FIGURE 2.** Transcript profiles of mitochondrial components in rice embryos germinated and grown under aerobic or anaerobic conditions. cDNA was prepared from rice embryo tissue 0, 1, 4, 12, 24, and 48 h after continuous growth in aerobic (red lines) or anaerobic (blue lines) conditions and used as a template in quantitative PCR assays to determine levels of various transcripts encoding mitochondrial components. A, using SOM clustering, the transcript profiles were classified into three groups. Group 1 included transcripts with aerobic and anaerobic profiles that did not group into the same cluster. Group 2 included transcripts with aerobic and anaerobic profiles that clustered together and either increased from low levels in dry seed (a) or showed relatively high levels in dry seed (b). For each group, an example of a profile for one of its members is provided, and for each time point the mean ± S.E. is shown (n = 3). The levels of Adh1 were also determined as a control for the treatments. Profiles of all transcripts listed can be found in the supplemental material. B, group 1 was further categorized into sets based on the timing of divergence between aerobic and anaerobic profiles. Set I showed differences after 1 h, Set II showed differences after 4 h, and Set III showed differences after 12 h. For each set, an example of a profile for one of its members is provided, and for each time point, the mean ± S.E. is shown for three independent replicate experiments.
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cantly affected by oxygen availability (Group 2). More specifically, Group 1 transcripts exhibited 2–10-fold increases after growth in an aerobic environment, whereas under anaerobic conditions, transcript abundance was maintained at levels similar to those seen in unimbibed (0 h) rice embryos. Notably, all of the oxygen-responsive transcripts were nuclear encoded with one exception, the mitochondrial encoded subunit of cytochrome c oxidase, \textit{cox2}. Although transcripts in Group 2 showed no significant response to oxygen, they differed in their profiles over time. Group 2a showed increases from relatively low levels in the dry seed (0 h) with maximum levels observed after 24 or 48 h, demonstrating that accumulation of some transcripts encoding mitochondrial proteins does occur under anaerobic conditions. Group 2b transcripts were relatively stable, with levels found to be similar in both unimbibed tissue (0 h) and after 48 h of growth under both conditions. As a control, transcript levels of alcohol dehydrogenase, \textit{Adh1}, a known and well studied anaerobically induced gene in rice, responded as expected, with message levels increasing rapidly after 12 h of growth in anaerobic conditions while remaining at similar levels in the presence of oxygen over the 48-h time period, essentially mirroring the changes seen for the oxygen-responsive transcripts (Group 1).

The transcripts showing a response to oxygen (Group 1) could be further categorized in terms of the timing of when the aerobic and anaerobic transcript profiles diverged (Fig. 2B). Sets I, II, and III indicate divergence after 1, 4, and 12 h, respectively, as defined by statistically significant differences between transcript levels under the different growth conditions at the same time point (determined using \textit{t} tests; \( p < 0.05 \)). Although the majority of transcripts examined showed differences after 4 h (Set II), a few notable exceptions were found, including \textit{Rps14} and \textit{Ant-1} (adenine nucleotide translocator) transcripts (Set I), which showed a rapid response to oxygen with a significant difference detected after just 1 h, and levels of \textit{cox2}, \textit{Adl2b}, and \textit{Ucp-2} (uncoupling protein) (Set III) transcripts, which showed a delayed response with significant differences not evident until after 12 h. Thus, it is possible to define a set of \textit{aerobic} genes that differ in the timing of their response to the presence of oxygen.

To further dissect the link between development and the timing of oxygen responsiveness, transcript analysis was conducted during the switch at 24 h between anaerobic and aerobic growth and \textit{vice versa}, again using quantitative reverse transcription-PCR (Fig. 3). The mitochondrial components were again categorized based on their response to oxygen availability using clustering algorithms. Group A showed large responses to changes in oxygen availability in that transcript profiles for anaerobic to aerobic switching and \textit{vice versa} could be assembled into different clusters based on SOM analysis (supplemental Fig. 2). In contrast, profiles of Group B transcripts were assembled into the same cluster, indicating no significant differences between the two growth regimes over the time period of the experiment. Although for some transcripts in Group B, a difference was observed at the 24 h time point, transcript levels under the different regimes were shown to rapidly converge after switching, revealing a lack of response to changes in oxygen availability (supplemental Fig. 2). For those transcripts showing a response to changes in oxygen availability (Group A, Fig. 3), if embryos initially grown under aerobic conditions were changed to anaerobic conditions, transcript levels declined to be similar to or below levels observed after anaerobic germination (2–5-fold decrease) after 24 h (\textit{i.e.} at the 48 h time point). Conversely, if oxygen was introduced to anaerobically grown rice, transcript abundance increased to levels seen for rice germinated in aerobic conditions (2–5-fold increase) within 24 h.

![FIGURE 3. Transcript profiles of mitochondrial components in rice embryos germinated under aerobic or anaerobic conditions and then switched to the other growth regime. cDNA was prepared from rice embryo tissue from dry seed (0 h), after 24 h of growth in aerobic (red) or anaerobic (blue) conditions and 1 (25 h), 4 (28 h), 12 (36 h), 24 (48 h), and 48 h (72 h) after switching to the other condition (solid line and solid squares, aerobic to anaerobic switch; dashed line and solid squares, anaerobic to aerobic switch) (the switch in growth conditions is indicated by a change in \textit{line color}). Quantitative PCR was used to determine levels of various transcripts encoding mitochondrial components. Using SOM clustering, the transcript profiles were classified into two groups. Group A included transcripts with aerobic and anaerobic profiles that did not group into the same cluster. Group B included transcripts with aerobic and anaerobic profiles that clustered together. For each group, an example of a profile for one of its members is provided, and for each time point, the mean ± S.E. is shown for three independent replicate experiments. The levels of \textit{Adh1} were also determined as a control for the treatments. Profiles of all transcripts listed can be found in the supplemental material.](image-url)
Generally, transcript levels responded rapidly to a change in oxygen conditions. Slightly quicker responses were seen for increases in abundance (usually 50% or greater in the first hour) compared with decreases (significant declines only seen 4 h after the switch). This may be due to the fact that the introduction of oxygen into the system is rapid compared with the slower complete removal of oxygen by continuous bubbling with nitrogen. Interestingly, using the switch approach, transcripts that were affected by changes in oxygen levels all showed relatively rapid responses. This is in contrast to the tiered response observed when oxygen was present during rice embryo germination (Fig. 2B), indicating that at later time points, environmental signals, in the form of oxygen availability, are exerting a greater influence over transcript abundance relative to developmental cues that appear to be important at earlier developmental stages. Nonetheless, categorization of genes showing a response to switching between aerobic and anaerobic conditions (Group A, Fig. 3) was consistent with those previously defined as aerobic genes (Group 1, Fig. 2).

Protein Levels of Mitochondrial Components Are Differentially Affected by Oxygen Availability—To determine if these oxygen-dependent transcriptional activations were leading to changes in the abundance of proteins in mitochondria isolated from rice embryo tissue grown under the different conditions, levels of selected proteins were quantified by immunodetection (Fig. 4). Quantitation was performed by normalizing to the sample with the highest level of protein observed and expressing the relative amount as a percentage. For germination and continuous growth in either aerobic or anaerobic conditions, levels of cytochrome c, RISP, the E1α subunit of PDH, and the E2 subunit of PDH and the oxoglutarate dehydrogenase complex were significantly affected by changes in oxygen availability. Protein amounts after growth under anaerobic conditions (48N) were only 8–36% of levels seen after growth under aerobic conditions (48A). The mitochondrial encoded cytochrome oxidase subunit, Cox2, showed a more moderate effect, with levels in mitochondria isolated from anaerobically grown tissue at 72% of those seen in mitochondria isolated from aerobically grown tissue. In contrast, levels of F1α, HSP60, HSP70, and voltage-dependent anion channel showed little or no difference between mitochondria isolated from tissue grown aerobically and anaerobically. Levels of the TOM20 showed a contrasting pattern. It was found to be 10-fold more abundant in mitochondria isolated from aerobically grown tissue compared with mitochondria isolated from aerobically grown tissue.

The Capacity of the General Mitochondrial Protein Import Pathway but Not the Carrier Import Pathway Is Affected by Oxygen Availability—The large differences observed in levels of TOM20 raises the question of the effect of this change on mitochondrial protein import capacity. Analysis of import capacity using isolated mitochondria from various developmental time points (30 min, 3 h, 12 h, and 24 h postimbibition) isolated from aerobically and anaerobically grown tissue was undertaken (Fig. 5). Precursors representing AOX (alternative oxidase) and the F1αd subunit of ATP synthase were used to assess the capacity of the general import pathway, whereas the phosphate carrier (PDC) and Tim23 precursors were used to assess the capacity of the carrier import pathway (29, 35–37).

All precursors were successfully imported into mitochondria isolated from tissue grown under aerobic and anaerobic conditions at the four time points. An example of one of these import experiments is shown in Fig. 5A, where mitochondria isolated from tissue grown for 12 h under both conditions was used. The amount of import was determined by measuring pixel density of the proteinase K-protected mature protein on the autoradiograph and then normalizing this to the amount of mitochondria isolated from tissue grown under aerobic conditions (Fig. 5B). This may be due to the fact that the introduction of oxygen into the system is rapid compared with the slower complete removal of oxygen by continuous bubbling with nitrogen. Interestingly, using the switch approach, transcripts that were affected by changes in oxygen levels all showed relatively rapid responses. This is in contrast to the tiered response observed when oxygen was present during rice embryo germination (Fig. 2B), indicating that at later time points, environmental signals, in the form of oxygen availability, are exerting a greater influence over transcript abundance relative to developmental cues that appear to be important at earlier developmental stages. Nonetheless, categorization of genes showing a response to switching between aerobic and anaerobic conditions (Group A, Fig. 3) was consistent with those previously defined as aerobic genes (Group 1, Fig. 2).

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The import reaction. Using this approach and performing each import in triplicate allowed an examination of changes in the capacity of both import pathways over early rice embryo development and in response to oxygen availability (Fig. 5B). Under normal aerobic conditions (red columns), it was found that the amount of import of AOX and F₆,₇ increased dramatically in 12 and 24 h samples, whereas generally, there was no significant difference between the four developmental stages examined (except at 3 h for Tim23). Interestingly, when mitochondria from anaerobically grown tissue (blue columns) was compared with mitochondria isolated from aerobic tissue, a clear difference in the amount of import of the precursors utilizing the general import pathway was observed, whereas no significant differences in the amount of import of precursors using the carrier import pathway were observed.

To further investigate these differences, import of a processed (AtRps10) and an unprocessed (LsRps10) form of the Rps10 precursor was assessed in mitochondria grown for 3 and 24 h under both growth conditions (red, aerobic; blue, anaerobic). The amount of import for each precursor was determined from pixel density measurements of the phosphorimaging plate using ImageGauge software and were normalized to allow comparison. C, the import of two forms of the ribosomal protein s10 (Rps10) was assessed in triplicate using mitochondria at each developmental stage (30 min, 3 h, 12 h, and 24 h) and from both growth conditions (red, aerobic; blue, anaerobic). The amount of import for each precursor was determined from pixel density measurements of the phosphorimaging plate using ImageGauge software and were normalized to allow comparison. D, import of AOX, F₆,₇, and P₇ precursors was assessed using mitochondria isolated from tissue grown for 48 h in both aerobic (red) and anaerobic (blue) conditions as well as tissue grown initially in anaerobic conditions for 24 h followed by reintroduction of oxygen for a further 24 h (purple). For all graphs presented, statistical significance (p < 0.05) was calculated using t tests. *, a significant difference between developmental stages; °, a significant difference between growth conditions for that developmental stage.
growth for 24 h. Import of AOX and Fαd was reduced after 48 h of growth in anaerobic conditions (48N) in line with results presented in Fig. 5B. However, import of AOX and Fαd recovered to aerobic levels when growth was switched from anaerobic to aerobic conditions at 24 h.

The Proteome of Mitochondria Isolated from Tissue Germinated and Grown under Anaerobic Conditions Reveals Subtle Differences in Protein Composition—To further analyze these multilayered effects of oxygen on mitochondrial biogenesis, we assessed the protein composition of mitochondria isolated from rice embryos germinated and grown without oxygen using two-dimensional gel electrophoresis (Fig. 6). Comparison of 48 h anaerobic samples with mitochondrial protein profiles after growth for 48 h under aerobic conditions highlighted a series of 13 protein spots that appeared to show differences in abundance (Table 1). -Fold changes in abundance were calculated over triplicate experiments, and these changes were tested for statistical significance using t-tests (p < 0.05). Protein levels found to be significantly higher or lower in mitochondria isolated from tissue grown under anaerobic conditions are indicated with boldface type and underlining, respectively (Table 1). This analysis revealed that three proteins from the TIM17/22/23 family were 6–14 times more abundant under anaerobic conditions (spots 11–13). This was similar to the large differences observed for TOM20 in Western blots of aerobic and anaerobic samples (Fig. 4). MPPα and MPPβ were detected as lower in abundance in anaerobically grown samples (spots 2, 4, and 6), consistent with the lower abundance of RISP (Fig. 4) and MPPα and MPPβ transcripts (Fig. 2). An isoform of HSP70, enzymes involved in carbon metabolism (E1α subunit of PDH, succinyl CoA ligase, and an isoform of mitochondrial aldehyde dehydrogenase), and a mitochondrial translation elongation factor were also found to be lower in abundance in anaerobic samples (spots 1, 3, and 7–9). Notably, although TIM17/22/23 proteins are imported via the carrier import pathway, the other proteins identified as decreased in abundance in anaerobic samples are normally imported via the general import pathway (Table 1, last column).

Together, the analysis of transcript levels (Fig. 2) and mitochondrial protein composition (Figs. 4 and 6, Table 1) indicate that transcriptional as well as post-transcriptional regulation is occurring. Changes in protein abundance for the E1α and E2 subunits of PDH, cytochrome c, and cox2 (Fig. 4) correlated with the oxygen dependence of their transcript induction (Fig. 2). The lack of response in Fα protein levels (Fig. 4) also correlated with the lack of oxygen response in transcript levels (Fig. 2). Another correlation was observed between the oxygen response of protein levels of RISP (Fig. 4) and the α and β subunits of MPP (Fig. 6, Table 1) and with the oxygen-dependent transcriptional response of the α and β subunits of MPP (Fig. 2; all components of the heme-containing cytochrome bc1 complex in the electron transport chain in plants). In contrast, although transcripts of TOM20, TOM40, TIM22, and TIM44 were unresponsive to oxygen availability (Fig. 2), protein abundance of import components determined by Western blotting (TOM20; Fig. 4) and two-dimnensional gel analysis (members of the TIM17/22/23 family; Fig. 6, Table 1) showed that they were all more abundant in mitochondria isolated from anaerobically grown tissue.

DISCUSSION

Is the Reduced Capacity of the General Import Pathway under Anaerobic Conditions due to Decreased Levels of MPP?—This study has revealed that the mitochondrial protein import path-
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### Table 1

Proteins identified following comparison of mitochondrial samples isolated from rice embryo tissue after growth under aerobic and anaerobic conditions for 48 h

| Description                                                                 | Spot | Accession | MP | Cov | MOWSE | MM gel | MM match | -Fold difference | Import pathway |
|----------------------------------------------------------------------------|------|-----------|----|-----|-------|--------|----------|------------------|----------------|
| Import                                                                     |      |           |    |     |       |        |          |                  |                |
| Import inner membrane translocase subunit                                  | 11   | Os03g19290| 7  | 41  | 203   | 22,000 | 18,470   | 7.9*            | CIP            |
| (TIM17/TIM22/TIM23 family)                                                 |      |           |    |     |       |        |          |                  |                |
| Import inner membrane translocase subunit                                  | 12   | Os03g19290| 13 | 48  | 295   | 22,000 | 18,470   | 13.8*           | CIP            |
| (TIM17/TIM22/TIM23 family)                                                 |      |           |    |     |       |        |          |                  |                |
| Putative stress-inducible membrane pore protein                            | 13   | Os01g19770| 5  | 15  | 149   | 19,000 | 28,052   | 5.8*            | CIP            |
| (TIM17/TIM22/TIM23 family)                                                 |      |           |    |     |       |        |          |                  |                |
| Mitochondrial processing peptidase β-chain                                 | 2    | Os03g11410| 31 | 44  | 1115  | 65,000 | 58,826   | 0.5*            | GIP            |
| Mitochondrial processing peptidase α-chain                                 | 4    | Os01g53700| 22 | 17  | 639   | 58,000 | 53,913   | 0.4*            | GIP            |
| Mitochondrial processing peptidase α-chain                                 | 6    | Os01g09560| 9  | 23  | 142   | 58,000 | 54,024   | 0.4*            | GIP            |
| Tricarboxylic acid cycle                                                    |      |           |    |     |       |        |          |                  |                |
| Putative pyruvate dehydrogenase E1 β subunit isomorph 1                     | 7    | Os09g33500| 5  | 14  | 126   | 40,000 | 40,337   | 0.6*            | GIP            |
| Putative succinyl-CoA ligase (GDP-forming) β-chain, mitochondrial precursor | 8    | Os02g10830| 10 | 24  | 170   | 41,000 | 45,063   | 0.7*            | GIP            |
| Chaperone                                                                  |      |           |    |     |       |        |          |                  |                |
| Putative heat shock 70-kDa protein, mitochondrial precursor                 | 1    | Os03g02260| 2  | 5   | 46    | 70,000 | 70,514   | 0.5*            | GIP            |
| Mitochondrial translation                                                   |      |           |    |     |       |        |          |                  |                |
| Translational elongation factor Tu                                           | 9    | Os03g63410| 6  | 17  | 97    | 42,000 | 48,504   | 0.3*            | GIP            |
| Other                                                                      |      |           |    |     |       |        |          |                  |                |
| Mitochondrial aldehyde dehydrogenase                                       | 3    | Os02g19720| 2  | 5   | 61    | 62,000 | 62,459   | 0.4*            | GIP            |
| ALDH2a                                                                     | 5    | Os06g15990| 9  | 19  | 207   | 58,000 | 59,270   | 2.0*            | GIP            |
| Putative late embryogenesis abundant protein                               | 10   | Os04g52110| 7  | 28  | 155   | 29,000 | 28,848   | 2.2*            | GIP            |

* t tests performed indicated that the differences observed were significant (p < 0.05).

In plants, MPP is integrated into the cytochrome bc1 complex of the respiratory chain (18, 19, 21). The capacity for import via the general import pathway correlated with the abundance of the α and β subunits of MPP. More specifically, protein import via the general import pathway using mitochondria isolated from anaerobically grown tissue was 2–4-fold lower compared with levels observed with mitochondria isolated from aerobically grown tissue (12 and 24 h; Fig. 5B), and protein abundance of the α and β subunits of MPP was also shown to be at least 2-fold lower (Table 1). Therefore, since the heme-containing bc1 complex containing the MPP is required for mitochondrial protein import, a case can be made for its low abundance in mitochondria from anaerobic tissue having an impact on protein import. Interestingly, data from microarray approaches that allow genome-wide identification of yeast aerobic genes reveal that the matrix-located α and β subunits of MPP (MAS1 and MAS2) are not significantly affected by changes in oxygen availability (6).

Initially, it may be postulated that if the level of MPP is regulating protein import capacity, this may explain why the general import pathway is more affected compared with the carrier import pathway, given that carrier proteins generally lack a cleavable presequence. However, in plants, carrier proteins, such as the adenine nucleotide translocator and P, contain a cleavable extension (35, 39, 40). We have previously shown that the N-terminal targeting signal of these proteins is required for efficient insertion into the inner mitochondrial membrane (41) and is removed in a two-step process, the first catalyzed by MPP and the second by an unknown intermembrane space protease (35). In addition, we have shown that import of an unprocessed...
protein via the general import pathway is not affected by oxygen (Fig. 5C). Thus, low levels of the cytochrome $bc_1$ complex are sufficient to support both the carrier and general import pathways, but a lack of oxygen impairs further accumulation of this complex and thereby prevents any increase in import capacity that occurs under normal growth conditions. Furthermore, the accumulation of MPP subunits is presumably facilitated by the observed increases in their corresponding transcripts. Interestingly, a recent analysis of the whole seed proteome in *Arabidopsis* examined proteins whose synthesis was inhibited by $\alpha$-amanitin, an inhibitor of RNA polymerase II (42). Some mitochondrial proteins were identified in this study, including MPP, indicating that de novo synthesis of the corresponding transcripts is required for protein accumulation of $\alpha$ and $\beta$ subunits of MPP during germination.

In fungal systems, it has been clearly demonstrated that there is no link between processing capacity and protein import competence, where inhibition of processing has been shown to have no effect on the ability of mitochondria to import proteins (43). However, in plant systems, the situation is unclear, with one study suggesting that a link between import and processing exists, using 1,10-phenanthroline and site-directed mutagenesis to inhibit processing (44), whereas another report suggested that there was no link based on evidence using a dihydrofolate reductase passenger protein linked to methotrexate (45). The results presented here suggest that levels of MPP and protein import capacity via the general import pathway are correlated. Thus, a link between processing and import may be dependent on the system under investigation. In most mitochondrial preparations, the abundances of respiratory chain complexes, such as the cytochrome $bc_1$ complex, are significantly higher than the abundance of the outer or inner membrane protein translocases, and thus, no limitations on processing would be observed. However, the abundance of import components in mitochondria of germinating rice embryos is relatively high, at similar or even higher levels than the respiratory chain complexes (25) (Fig. 6). Thus, in such situations the abundance of MPP appears to limit protein import capacity.

A recent study in yeast provides evidence for physical interaction of the translocase of the inner membrane with the $bc_1$ and cytochrome $c$ oxidase complexes of the respiratory chain (46). Whether such an association exists in plant mitochondria is unknown, but if it does occur in addition to the plant-specific link between the $bc_1$ complex and MPP, it would indicate a close connection between components of two major mitochondrial functions.

**Oxygen Availability Differentially Affects Gene Expression of Mitochondrial Components at Both Transcript and Protein Levels**—Transcripts encoding components of the respiratory chain (subunits of complexes I, IV, V, and the mobile electron carrier, cytochrome $c$), adenine nucleotide translocator, subunits of PDH, ribosomal proteins, and the $\alpha$ and $\beta$ subunits of MPP all showed a response to oxygen availability with an increase in transcript levels in the presence of oxygen and a repression of this increase in the absence of oxygen. Comparing transcript profiles of different oxygen-responsive genes revealed that some genes respond very quickly to the presence of oxygen (e.g. *Ant-1*), suggesting that oxygen availability is sensed early in rice embryo development. Furthermore, rapid changes in transcript abundance of these *aerobic* genes were seen when samples were transferred from aerobic to anaerobic conditions (Fig. 3), with an immediate decline in transcript abundance observed. In yeast, similar decreases of transcript abundance over short time periods have been shown to require active transcript degradation, since a stop in transcription alone is not sufficient to account for such rapid depletions (47). This suggests that oxygen-dependent gene expression and its involvement in mitochondrial biogenesis is a complex process involving both transcriptional and post-transcriptional mechanisms. Furthermore, the change in *Adh1* transcript levels seen only after 12 h, during germination under anoxic conditions (Fig. 2), indicates that the induction of hypoxic genes may be downstream of the repression of aerobic genes. A comparable situation exists in yeast, where the Hap1 transcription factor promotes transcription of aerobic genes but also activates transcription of *Rox1* and *Mot3*, which then subsequently act as transcriptional repressors of hypoxic genes (48).

Interestingly, transcripts of the nuclear encoded genes unaffected by oxygen levels included import components (*TOM20, TOM40-1, TIM44*, and *TIM22-1*) and the mitochondrial RNA polymerase (*RpoTm*; Figs. 2 and 3). Other transcripts that were relatively unresponsive to oxygen included mitochondrial encoded genes representing subunits of complexes I, III, and V of the electron transport chain. This is in general agreement with the findings of Tsuji et al. (60), where transcripts of nuclear encoded respiratory genes but not mitochondrial encoded respiratory genes, were markedly reduced in rice seedlings during the first 12 h of hypoxia. Since the mitochondrial encoded transcripts are relatively unaffected by changes in oxygen levels, how is their expression coordinated with nuclear genes encoding subunits contributing to the same complex? Using the cytochrome $bc_1$ complex as an example, transcript levels of the mitochondrial encoded subunit *cob* do not differ dramatically in response to anaerobic conditions (Figs. 2 and 3, supplemental Figs. 1 and 2). In contrast, protein levels of the nuclear encoded subunits of the cytochrome $bc_1$ complex (RISP, MPP$\alpha$, and MPP$\beta$) were all down-regulated in response to oxygen deficit (Figs. 4 and 6). Although cob protein levels have not been determined, down-regulation of mitochondrial translational elongation factor Tu (Fig. 6, Table 1) and transcripts of the mitochondrial ribosomal protein, *Rps14* (Figs. 2 and 3, supplemental Figs. 1 and 2) indicates that regulation may be exercised at the level of mitochondrial translation to coordinate nuclear and mitochondrial gene expression and ensure correct subunit stoichiometry of mitochondrial complexes.

One exception to the observation that mitochondrial encoded genes were not affected by changes in oxygen was seen for subunit 2 of the cytochrome $c$ oxidase complex (cox2), which was found to respond to oxygen in a similar way to the nuclear encoded *aerobic* genes (Figs. 2 and 3). Mitochondrial encoded genes are widely considered not to be under transcriptional regulation but instead are regulated by a variety of post-transcriptional events (49, 50). Since quantitative reverse transcription–PCR quantifies steady state mRNA levels, which represent the balance between transcription and mRNA stability, it is possible that the stability of the cox2 transcript may be
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changing in response to oxygen availability, and numerous studies provide evidence for this type of regulation of mitochondrial encoded genes (51–54). Since this study has also determined that transcript levels of the mitochondrial RNA polymerase (RpoTm) are unresponsive to oxygen levels and that transcript levels of other mitochondrial encoded components are relatively unaffected by oxygen deficit (Fig. 2, supplemental Fig. 1), this indicates that some form of post-transcriptional regulation may explain the observed changes in cox2 transcript levels. This appears to differ from the situation in yeast, where levels of cox2 are regulated at the translational level under low oxygen conditions (55), but is similar to observations in mammalian cells showing that cox2 transcript levels respond to changes in oxygen availability (56). Furthermore, cox2 protein levels were found to change significantly compared with other mitochondrial encoded proteins in response to sucrose starvation of Arabidopsis cell culture (49), indicating that regulation of cox2 independent of other mitochondrial encoded components exists in other systems.

In addition, in the present study, cox2 transcripts showed a lag in response to the introduction or removal of oxygen compared with the nuclear encoded oxygen-responsive transcripts. For the nitrogen to air switch, an increase was observed for other transcripts 1 h after switching (at 25 h), whereas for cox2, transcripts only increased after 4 h (at 28 h). For the air to nitrogen switch, a decrease was seen after 4 h for most oxygen-responsive transcripts, whereas a decrease in cox2 message abundance was only observed 12 h after the switch (at 36 h). This difference in timing suggests that changes in mitochondrial gene expression in response to oxygen availability may be downstream in a signaling pathway from changes in nuclear gene expression.

Finally, a late embryogenesis abundant (LEA) protein was identified from two-dimensional gel analysis (Fig. 5, Table 1) that was found to be at higher levels in mitochondria isolated from anaerobically grown tissue compared with mitochondria isolated from dry seed and from aerobically grown tissue. Interestingly, this LEA protein is different from the rice LEA identified previously as highly abundant in mitochondria isolated from unimbibed rice embryos (25) but is also predicted to be located in mitochondria. A recent study suggests a role for a mitochondrial LEA protein in protecting stored mitochondrial proteins during desiccation in pea (57), and LEA proteins have been found to be induced in response to other stress conditions (58). The LEA protein identified here may also play some kind of protective role during oxygen deficit and, to our knowledge, is the first LEA protein to be found to be up-regulated in response to oxygen deficit.

Conclusions and Future Perspectives—Rice is one of only a few multicellular eukaryotic organisms that can survive and grow in the absence of oxygen and provides an opportunity to differentiate oxygen signals and developmental cues required for mitochondrial biogenesis. The present study has (i) identified plant aerobic genes, revealing that as in yeast, many of these are components of the respiratory chain, which notably, in plants, includes the subunits of MPP; (ii) revealed that a lack of oxygen represses the normal increase in mitochondrial protein import observed during aerobic germination and suggests that a mechanistic link between protein import capacity and respiration, in the form of the bifunctional cytochrome bc1 complex, is central to these changes; and (iii) identified a system that can allow dissection of the pathway involved in oxygen signaling in plants, as well as ultimately identify the master switches involved, which occupy the roles of the heme activator protein transcription factors in yeast, for which no orthologues exist in plants.

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