The impact of impaired mitochondrial function on retrograde signalling: a meta-analysis of transcriptomic responses

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Abstract

Mitochondria occupy a central position in cellular metabolism. Their protein complement must therefore be dynamically adjusted to the metabolic demands of the cell. As >95% of mitochondrial proteins are encoded by nuclear DNA, regulation of the mitochondrial proteome requires signals that sense the status of the organelle and communicate it back to the nucleus. This is referred to as retrograde signalling. Mitochondria are tightly integrated into the network of cellular processes, and the output of mitochondrial retrograde signalling therefore not only feeds back to the mitochondrion, but also regulates functions across the cell. A number of transcriptomic studies have assessed the role of retrograde signalling in plants. However, single studies of a specific mitochondrial dysfunction may also measure secondary effects in addition to the specific transcriptomic output of mitochondrial signals. To gain an improved understanding of the output and role of mitochondrial retrograde signalling, a meta-analysis of 11 transcriptomic data sets from different models of plant mitochondrial dysfunction was performed. Comparing microarray data from stable mutants and short-term chemical treatments revealed unique features and commonalities in the responses that are under mitochondrial retrograde control. In particular, a common regulation of transcripts of the following functional categories was observed: plant–pathogen interactions, protein bio-synthesis, and light reactions of photosynthesis. The possibility of a novel mode of interorganellar signalling, in which the mitochondrion influences processes in the plastid and other parts of the cell, is discussed.

Key words: Arabidopsis, electron transport chain, microarray, mitochondria, respiration, retrograde signalling, ROS.

Introduction

Plant mitochondria occupy a central role in sustaining cellular ATP supply as well as in a whole variety of other metabolic processes, many of which involve multiple subcellular compartments (reviewed in Sweetlove et al., 2010; Millar et al., 2011). In particular, the processes of respiration in the mitochondrion and photosynthesis in the chloroplast are intimately linked (Raghavendra and Padmasree, 2003; Matsuo and Obokata, 2006; Rasmusson and Escobar, 2007; Nunes-Nesi et al., 2008, 2011). Alterations of mitochondrial respiratory metabolism can have dramatic effects on photosynthesis (Carrari et al., 2003; Dutilleul et al., 2003a, b; Nunes-Nesi et al., 2005). The coordination of organelar functions requires dynamic adjustment of gene expression by retrograde signalling, during which organelar stimuli regulate nuclear-encoded genes (Butow and Avadhani, 2004; Leister, 2005; Rhoads and Subbaiah, 2007). Retrograde control is necessary as the nucleus encodes most organelar proteins and therefore initially controls most aspects of organelar biogenesis and function. Due to the multitude of organelar functions, a variety of interlinked retrograde pathways can be expected (Leister, 2005). The extent to which different signals can be integrated into common pathways is not clear (Ho et al., 2008).

The first work on mitochondrial retrograde signalling was performed in yeast (Liao and Butow, 1993). Currently, different types of signalling molecules and pathways...
photosynthetic gene expression (Koussevitzky et al., 2005; Ramanathan and Schreiber, 2009; Woo et al., 2009). It is likely that the basic mitochondrial signalling strategies follow similar principles in plants.

Important progress has been made in the past two decades towards understanding plastid-to-nucleus signalling in plants (reviewed in Pesaresi et al., 2007; Woodson and Chory, 2008; Kleine et al., 2009). However, only little is known about the retrograde signalling pathway(s) from the mitochondria to the nucleus (Nott et al., 2006; Rhoads and Subbaiah, 2007). Mitochondrial alternative oxidase 1 (AOX1a) was the first nuclear gene that was shown to be retrograde regulated and mainly in the context of the response to stress (Vanlerberge and McIntosh, 1994, 1996; Djajanegara et al., 2002; Gray et al., 2004; Dojcinovic et al., 2005). Recently, the transcription factor (TF) ABI4 was identified as a repressor of AOX1a which is de-repressed under rotenone treatment (Giraud et al., 2009). ABI4 is also a regulator of plastid retrograde signalling to repress photosynthetic gene expression (Kousevitzky et al., 2007; Woodson and Chory, 2008). Thus both retrograde signalling pathways may feed into the abscisic acid (ABA) signalling pathway, the actual sensors within the organelle and second messengers of the signalling cascade represents one of the downstream components of a mitochondrial retrograde signalling pathway, the actual sensors within the organelle and second messengers of the signalling cascade are still unknown.

Several recent studies have compared publically available microarray data, to analyse the regulation of organellar genes (nuclear encoded or organellar encoded) under various stress conditions (Van Aken et al., 2009; Leister et al., 2011). However, organelles are tightly connected in the network of cellular processes, and changes in their biochemistry will have an impact at the whole-cell level. Therefore, not only nuclear-encoded organellar genes can be regulated in response to retrograde signals, but also genes associated with any cellular location and function. With the aim of unravelling common targets and pathways that are regulated by modulations in mitochondrial status, a meta-analysis of 11 transcriptomic data sets in which respiratory function was impaired in different ways has been performed.

Materials and methods

Plant materials

All Arabidopsis thaliana lines used in this study were the Columbia ecotype. PrxII F-KO and pOpOFF2(kan)::MSD1 have been described previously (Finkemeier et al., 2005; Schwarzländer et al., 2011). For all experiments, surface-sterilized seeds were plated on 0.7% (w/v) agar plates supplemented with half-strength Murashige and Skoog (MS) medium.

Seedlings were grown in a controlled growth chamber with a 14/10 h day/night cycle (21/19 °C) at a light intensity of 50 μmol m⁻² s⁻¹. Rosette tissue was used for the experiments at an age of 3 weeks.

Effectors treatments

Antimycin A (AA) and dexamethasone were purchased from Sigma-Aldrich (Gillingham, Dorset, UK). Stock solutions were prepared in ethanol (EtOH; AA) or dimethylsulphoxide (DMSO; dexamethasone). For treatments, stocks were diluted in 0.01% Tween-20 to 20 μM (AA) and 10 μM (dexamethasone) final concentrations, respectively. Effectors were sprayed using an atomizer (AA) or painted (dexamethasone) onto plants. For AA treatments, controls were performed with 0.01% Tween-20 and the appropriate amount of solvent (EtOH). Seedlings were dark-adapted for 2 h prior to treatments and were kept in the dark for the duration of the AA treatment.

Microarray experiments

For microarray experiments, rosette material was harvested and flash-frozen in liquid nitrogen. Total RNA was extracted using TRIZOL reagent (Invitrogen Ltd, Paisley, UK). cDNA preparation and labelling was carried out using the 3DNA Array 50 kit (version 2, Genisphere Inc., Hatfield, USA) according to the manufacturer’s instructions. Cy3- or Cy5-labelled cDNA probes were hybridized against 29k Arabidopsis Oligonucleotide Microarrays (http://ag.arizona.edu/microarray). Four biological replicates were analysed including two inversions of the labelling dye to avoid bias. Microarrays were scanned using an Affymetrix 428 Array scanner and acquisition software according to the manufacturer’s instructions. After scanning, images were analysed in GenePix Pro Version 4.1 software ( Molecular Devices, Sunnyvale, CA, USA) checking each spot individually. Raw data were saved as a.gpr-file and converted into a.mev-file using the Express Converter software (version 1.9, Dana Faber Cancer Institute, Boston, MA, USA). Data were normalized using the lowest (locfit) algorithm and block normalization in the MIDAS software (version 2.19, Dana Faber Cancer Institute, Boston, MA, USA). Normalized data were quality controlled in a spreadsheet file, and elements with median intensity <2 SDs from the mean of all median background values were discarded; in a case where one channel was discarded due to this criterion, but the other was >10 SDs from the background, the element was retained; duplicates in the data set were removed by averaging intensity values, and ratio data were generated using the FiRe macro (Beckers and Conrath, 2006; Garcia et al., 2006). The data sets were deposited at the EBI ArrayExpress database (accessions E-TABM-64, -1196, and -1197) according to the MIAME guidelines.

Microarray data of the published experiments were downloaded as processed files from the EBI ArrayExpress database (www.ebi.ac.uk) or the Gene Expression Omnibus database (GEO, http://www.ncbi.nlm.nih.gov). Experimental details and accession numbers can be found in the original publications as indicated in Table 1. Due to the heterogeneity of the experimental set-up of the different integrated experiments (such as sufficient numbers of replicates and microarray platforms), no careful statistical analysis could be performed. Instead microarrays were all analysed after the following criteria: only transcripts that were present in at least three replicates (two in cases where only two data sets were available) with a coefficient of variation <50% were used. Relative expression values were calculated and expressed as log2-transformed mean ratio. Hierarchical cluster analysis was performed using the algorithm for average linkage clustering with a Pearson correlation integrated in the MeV v.4.7.3 microarray software suite (Saeed et al., 2006). Functional class scoring was implemented using MapMan software (Usadel et al., 2005) applying the Hochberg correction. Co-expression networks were analysed using the Atted-II webinterface (Obayashi et al., 2011).
Table 1. Overview of microarray experiments used in the meta-analysis: for each, the mitochondrial target is indicated. The numbers in parentheses indicate the type of mitochondrial impairment as shown in Fig. 1. Transgenic lines, tissues, treatments, microarray platforms, and source publications are indicated. n = number of replicates.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Mitochondrial target</th>
<th>Genetic background</th>
<th>Plant tissue</th>
<th>Treatment</th>
<th>Time points</th>
<th>n</th>
<th>Microarray platform</th>
<th>Publication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antimycin A</td>
<td>Inhibitor of complex III (3)</td>
<td>WT (Col)</td>
<td>Rosettes, 3 weeks</td>
<td>20 μM antimycin A</td>
<td>2 h</td>
<td>4</td>
<td>Galbraith 29k</td>
<td>This study</td>
</tr>
<tr>
<td>Antimycin A (pndf F)</td>
<td>Inhibitor of complex III (3)</td>
<td>pnxl F (Col)</td>
<td>Rosettes, 3 weeks</td>
<td>20 μM antimycin A</td>
<td>2 h</td>
<td>4</td>
<td>Galbraith 29k</td>
<td>This study</td>
</tr>
<tr>
<td>aox1a</td>
<td>Loss of alternative oxidase (2)</td>
<td>aox1a (SALK_084897) (Col)</td>
<td>Leaves, 4 weeks</td>
<td>None</td>
<td>–</td>
<td>2</td>
<td>Affymetrix ATH1</td>
<td>Giraud et al. (2008)</td>
</tr>
<tr>
<td>uATPase9</td>
<td>Loss of mitochondrial ATP synthase (4)</td>
<td>AP2u-ATP9, A0u-ATP9</td>
<td>Flowers stage 12</td>
<td>None</td>
<td>–</td>
<td>4</td>
<td>Galbraith 29k</td>
<td>Busi et al. (2011)</td>
</tr>
<tr>
<td>msd1-RNAi</td>
<td>Loss of MSD1 protein (6)</td>
<td>pOpOFF2(kan):MSD1, pOpOFF2(kan):LUC (Col)</td>
<td>Rosettes, 3 weeks</td>
<td>None (10 μM dexamethasone for RNAi induction)</td>
<td>12 d</td>
<td>4</td>
<td>Galbraith 29k</td>
<td>This study</td>
</tr>
<tr>
<td>msh1×recA</td>
<td>Mitochondrial genome rearrangement (5)</td>
<td>msh1×recA3 (Col)</td>
<td>Aboveground tissue, 8 weeks</td>
<td>None</td>
<td>–</td>
<td>2</td>
<td>Affymetrix ATH1</td>
<td>Shedge et al., (2010)</td>
</tr>
<tr>
<td>Complex I</td>
<td>Loss of complex I (1)</td>
<td>ndufs4, ndufs1 (Col)</td>
<td>Leaves, 6 weeks</td>
<td>None</td>
<td>–</td>
<td>3</td>
<td>Affymetrix ATH1</td>
<td>Meyer et al. (2009)</td>
</tr>
<tr>
<td>Oligomycin</td>
<td>Inhibition of ATP-synthase (4)</td>
<td>WT cell culture (Ler)</td>
<td>Cell culture, 3 d light</td>
<td>1.25 μM oligomycin</td>
<td>3 h</td>
<td>2</td>
<td>Affymetrix ATH1</td>
<td>Clifton et al. (2005)</td>
</tr>
<tr>
<td>Rotenone</td>
<td>Inhibition of complex I (1)</td>
<td>WT cell culture (Ler)</td>
<td>Cell culture, 4 d light</td>
<td>40 μM rotenone</td>
<td>3 h</td>
<td>2</td>
<td>Affymetrix ATH1</td>
<td>Clifton et al. (2005)</td>
</tr>
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</table>

WT, wild type; Col, Columbia; Ler, Landsberg erecta; kan, kanamycin.

**Results and Discussion**

**Mitochondrial dysfunction induces transcript changes in diverse transcriptome data sets**

Eleven transcriptome data sets were selected that had been generated from various *Arabidopsis* tissues and ages (Table 1) in which either mitochondrial function was impaired genetically or the mitochondrial respiratory chain was inhibited at different points by short-term chemical treatment (Table 1, Fig. 1). Short-term application of respiratory inhibitors has the advantage of avoiding pleiotropic or acclimation responses which are observed in stable mutants. However, a possible drawback of using inhibitors is the degree of uncertainty about specificity of the agent, and special care must be taken in the design of experiments to use adequate concentrations, conditions, and controls. By analysing data sets from both types, the aim was to reveal commonalities in the response of functional gene groups a class sets for complex III inhibition by AA in the wild-type (WT) background and in the peroxiredoxinII F (prxII F) knockout mutant (Finkemeier et al., 2005).

Because of the heterogeneity of the experimental systems/conditions and microarray platforms used, no valid statistical comparison of the array data sets could be performed (Gadjev et al., 2006). Instead, transcriptomic data sets were compared on the basis of the relative fold changes (mutant/WT or treatment/control). Comparable data sets were generated using stringent criteria on the coefficient of variation of replicates to calculate the fold changes (log2); transcript data were only included in the analysis if they were present in at least three biological replicates (two in cases where only two replicates were available) and if the coefficient of variance was <50% (Supplementary Table S1 available at JXB online). A cut-off value of ±0.5 (log2) was used to define a transcript as regulated. Higher cut-off values can give more robust results on single transcripts, but they can overlook milder but coordinated changes of functionally related transcript groups that are biologically highly relevant (e.g. Leakey et al., 2009). Furthermore, for all data sets the same three-step strategy of analysis was used: (i) in a hierarchical cluster analysis unique transcripts were revealed as a measure for specificity of a certain mitochondrial dysfunction; (ii) the identified transcripts were analysed in a co-expression analysis with 1388 microarrays using the network drawer function of Aatted-II to assess linked regulation of the selected transcripts and to extrapolate the functional output of the perturbed signalling pathway; and (iii) to reveal commonalities in the response of functional gene groups a class
scoring algorithm with Hochberg correction, as implemented in the MapMan software, was applied for whole data sets.

Table 2 shows the number of transcripts which changed >0.5-fold (log2 ratio) in all 11 data sets as well as the number of overlapping transcripts between two respective experiments. While six of the data sets (aox1a, mshl×recA3, ndufa1, ndufs4, oligomycin, and rotenone) contained ~3000–4000 transcripts passing the threshold of 0.5-fold regulation, five data sets contained <1000 including the authors’ own three data sets [AA (WT), AA (prxII F), and msl4] with only 30–100 regulated transcripts. Even though the total number of overall detected transcripts from the authors’ own data sets using the 29K Galbraith arrays was only about half of the amount of those detected on the Affymetrix chips, the percentage of regulated transcripts was still much less (0.5–1.6% of all detected transcripts regulated in comparison with 18–29%; see Table 2). Importantly, this was not due to an experimental artefact from using the 29K Galbraith array system, as ~9–14% of all transcripts were differentially regulated in the u-ATP9 mutants which used the same array platform (Busi et al., 2009). Furthermore, this array system was used in a previous study revealing drastic effects on transcript abundances after short-term menadione treatments which provoked oxidative stress in Arabidopsis roots (Lehmann et al., 2009). The small transcript changes therefore represent a genuinely milder transcript response compared with the other systems, which most probably reflects the absence of a general stress or acclimation.

The 11 microarray data sets can be grouped according to their physiological effects on mitochondrial functions (Fig. 1).

(i) Complex I

Three data sets assessed defects of complex I on transcript levels. Complex I was affected in abundance in the ndufs4l mutant leaves (Meyer et al., 2009), and its activity was inhibited by 3 h short-term treatment of autotrophic cell cultures with 40 μM rotenone (Clifton et al., 2005), respectively. Thus, similarities in transcriptional regulation observed in these studies which were not observed in the other studies may result from a retrograde signal connected with complex I activity. A total of 24 and 27 transcripts were up- and down-regulated, respectively, exclusively in this system (0.5-fold; Supplementary Table S1 at JXB online). The connection between these transcripts was analysed using the network drawer of Atted II. Interestingly, 14 of the up-regulated transcripts [including alternative NADPH dehydrogenase 2 (NDH2, Atg22990), S-locus lecin protein kinase (At1g1330, At1g61360), downy mildew resistant 6 (DMR6, At5g24530), nodulin MtN-21-related (At3g36620), chitinase (At4g01700), and resistant to P. syringae 4 (RPS4, At5g45250)] were co-expressed in a network together with 144 transcripts, many of which are related to plant–pathogen interactions such as WRKY33 (At2g39470), MKK2 (At4g29810), MEK1 (At4g26070), MPK4 (At4g01370), MAPK10 (At4g08470), BAK1 (At4g33430), NPR1 (At1g64280), and PAD4 (At4g32430) (Supplementary Fig. S1). NPR1 and PAD4 are known regulators of the salicylic acid (SA)-dependent defence pathway, and PAD4 was reported to be involved in AOX1a-dependent transcript regulation (Ho et al., 2008). Twelve other up-regulated transcripts could be assigned to smaller co-expression networks that are closely linked to the large network and are related to biosynthesis of secondary metabolites, stilbenoid, diarylethannoid, and ginsenol biosynthesis, and glutathione metabolism, respectively. From 27 down-regulated transcripts, 18 [including phytochrome A (At1g09570), β-glucosidase 40 (At1g25650), WNK7 protein kinase (At1g49160), cellulose synthase 2 (CESA2, At4g39350), LRR-protein (At3g17640), VTC4 (At3g02870),

Fig. 1. Overview of microarray experiments dealing with mitochondrial impairments. Numbers depict treatments or mutations affecting mitochondrial targets. (1) Complex I: ndufa1, ndufs4, and rotenone. (2) AOX: aox1a. (3) Complex III: antimycin A. (4) Complex V: AP3- uATP9, AP9-uATP9, and oligomycin. (5) mtDNA: msh1×recA4. (6) MnSOD: msl4. (7) PeroxiredoxinII F: prxl F. The corresponding publications are given in Table 1. The hierarchical cluster analysis of 122 transcripts coding for mitochondrial electron transport proteins was performed using the algorithm for average linkage clustering with a Pearson correlation integrated in the MeV v.4.7.3 microarray software suite (Saeed et al., 2006). Yellow and blue indicate up- and down-regulated transcripts (more than ±0.5 log2 fold) in transgenic or treated plants in comparison with wild-type or untreated transgenic plants, respectively. Grey corresponds to missing values, and black indicates no regulation. The dendrogram on the top indicates the relationship of samples across the displayed transcripts.
methyltransferase MTA-70 family protein (At1g19340), and an AP2-domain transcription factor (At1g21910) were part of a large co-expression network with 170 more genes that are related to light signalling, cold-regulated genes, plant-pathogen interactions, cyanobioacid metabolism, the cell wall, and cytoskeleton-related elements which are part of a phagosome (beta 6, 5, and 8-tubulin). The strong interaction of induced and repressed transcripts in large co-expression networks suggests a regulatory pathway related to biotic defence, which seemed to be triggered as a result of complex I dysfunction (for a review on the role of mitochondrial biotic defence, see Amirsadeghi et al., 2007).

A recent study by Gleason et al. (2011) provided genetic proof that complex II of the mitochondrial electron transport chain (ETC) is associated with mitochondrial reactive oxygen species (ROS) production and the propagation of plant stress and defence responses. A higher electron flux through complex II in the complex I mutant, which also showed increased ROS levels (Meyer et al., 2009), may actually trigger the observed transcriptomic response. Furthermore, the early stress defence marker gene GSTF8, which was used in the study by Gleason et al. (2011), was strongly induced after rotenone treatment. As the transcriptomic data from the complex II mutant (Gleason et al., 2011) were only available after completion of this analysis, they were not included here.

(ii) AOX

Giraud et al. (2008) used a knock-out mutant of the AOX1a gene which contained no immunodetectable AOX protein to generate a transcriptomic data set. AOX is part of an alternative respiratory pathway specific to plants as well as certain fungi and protists, and by-passes complex III as an electron acceptor from ubiquinol. It acts as an alternative terminal oxidase which reduces oxygen to water without the build up of a proton gradient (Vanlerberghe and McIntosh, 1997). AOX function is particularly important under stress conditions to avoid over-reduction of the ubiquinone pool and increased ROS generation (Maxwell et al., 1999). However, even under non-stress conditions, the AOX makes up ~20–30% of the total cellular respiratory capacity (Gray et al., 2004), and changes in transcript abundance could therefore result from a retrograde signal triggered by a higher flux through the cyanide-sensitive pathway of the ETC or increased coupling of respiration and ATP synthesis. Consequently, a loss of function in AOX may be expected to cause defects opposite to those of other dysfunctional respiratory complexes. In the aox1a mutant, 624 and 1179 transcripts were specifically up- or down-regulated, respectively (0.5-fold log2 threshold) (Supplementary Table S1 at JXB online). Those 1803 transcripts were from various functional gene classes, with a high representation of cell wall and lipid metabolism, biotic and abiotic stress, polyamine metabolism, degradation of xenobiotics, C1 metabolism, RNA regulation of transcription, and protein synthesis. Giraud et al. (2008) reported a significant change of 2985 transcripts for this mutant in comparison with the WT at a similar fold change level as used in this meta-analysis (2944 were detected based on the criteria used), which supports the validity of the analysis. Thus, the loss of AOX1a resulted in a specific transcript response that was mostly independent or even inversely regulated in comparison with the other data sets. This may suggest that the particular path of electron flow through the ETC is critical in mounting the underlying signal.

(iii) Complex III

Two transcriptomic data sets were generated assessing the effect of complex III inhibition. AA treatment for 2 h partly inhibited respiration, as measured by reduction of oxygen uptake by one-third (Schwarzländer et al., 2009). AA inhibits complex III by binding to the ‘N’ site, strongly stimulating superoxide release (Murphy, 2009). To unravel
The rationale for including this data set was that responses to mitochondrial ROS (mtROS; specifically H₂O₂) are expected to be more pronounced in AA-treated prxII F plants than in AA-treated WT plants. In contrast, alteration of mitochondrial energy metabolism, as a result of AA treatment, can be expected to be similar in both AA-treated WT and prxII F mutants.

The transcriptomic changes detected in the prxII F mutant background (109 transcripts) relative to the WT (36 transcripts) were indeed in line with the expectation of a stronger response in the absence of PrxII F. However, both were relatively subtle compared with the other inhibitor experiments (Table 2). As AA is known to induce programmed cell death upon prolonged exposure, an early time point had been selected to generate the data sets, before major stress responses, such as electrolyte leakage, lipid peroxidation, and glutathione oxidation, were observed (in both the mutant and WT; unpublished data). The fact that more transcripts were regulated in the prxII F mutant background after AA treatment compared with the WT is consistent with ROS release upon AA treatment being responsible for the majority of transcriptomic changes rather than the inhibited flux through complex III (and the cyanide-sensitive pathway in general, see above, for AOX).

Transcriptomic data of the response of Arabidopsis leaves to AA have been published previously by Yu et al. (2001), and selected data were published by Rhoads and Subbaiah (2007) using AA treatment for 6 h. Both studies identified ROS generation as the dominant effect of AA on transcript changes. However, Yu et al. (2001) described 621 transcripts which were doubled in abundance after 30 min of AA treatment, whereas in the authors’ arrays none of the transcripts was increased by >2-fold. This most probably reflects the experimental design in the study of Yu et al. (2001) in which AA treatment was administered by floating detached leaves on an AA solution, while control leaves were sampled directly from the plant. Thus, many of the observed changes in transcripts could be a result of tissue wounding and reduced gas exchange during floating rather than due to AA.

In the AA data sets (prxII F and WT background), 45 transcripts were induced that were not regulated in any of the other nine data sets. Twenty-one of these 45 transcripts were found in the Atted-II co-expression database and 15 of those were connected with 174 more transcripts in a large co-expression network (again including PAD4, NPR1, BCS1, WRKY33, and WRKY 46) (Supplementary Fig. S1 at JXB online). Within this network, transcripts encoding proteins involved in secondary metabolism, plant–pathogen interaction, phenylalanine, tyrosine, and tryptophan biosynthesis, SNARE interactions in vesicular transport, cysteine and methionine metabolism, and ethylene and calcium signalling were detected.

(iv) Complex V

Three data sets included here dealt with the response to reduced mitochondrial ATP synthase (complex V) activity. Complex V activity was inhibited either by short-term oligomycin treatment (2 h) in autotrophic cell cultures (Clifton et al., 2005), or by expression of the unedited form of the ATP synthase subunit 9 in Arabidopsis flowers under the control of APETALA 3 (AP3) and the A9 promoter, respectively (Busi et al., 2011). While no effects on cellular respiration were observed after 24 h of oligomycin treatment, the respiration rate was decreased by 50–65% and ATP levels by 35% in flowers of the u-ATP9 lines (Busi et al., 2011). However, in a different study, short-term oligomycin treatment (1 h, 10 μM) also resulted in a lowered ATP content (by 30%) in tobacco suspension cultures (Wakamatsu et al., 2010). Similar transcript changes between these three data sets thus might have been triggered by a lowered ATP content. Only five transcripts were exclusively regulated by complex V inhibition (four induced, one repressed) and not in the other data sets (Supplementary Table S1 at JXB online). Only two of these transcripts, one encoding an unknown protein (At5g47940) and one an armadillo/beta-catenin repeat family protein (At3g01400), were connected with 10 other transcripts [including MKP1 (At3g55270) and bHLH-TF (At5g46760)] in a co-expression network (Supplementary Fig. S1). The low number of transcripts exclusively regulated by complex V dysfunction may suggest that only a low number of transcripts was regulated by inhibition of complex V activity and thus compromised ATP levels. However, a more likely explanation is that the poor overlap resulted from the use of different tissues (cell cultures and flowers) in the two studies. Moreover, ATP levels were most probably perturbed in several of the other experiments as a result of impaired mitochondrial function, such as in the ndufs4 mutant (Meyer et al., 2009).

(v) mtDNA

One data set analysed in this study was generated from the double mutant msh1×recA3 that shows severely compromised recombination surveillance of the mitochondrial genome (Shedge et al., 2010). This mutant shows extensive rearrangements in its mitochondrial DNA (mtDNA) along with severely affected development and increased thermotolerance. As mitochondrial-encoded transcript levels were strongly up-regulated in the mutant and many of the recombination sites are within the open reading frames for mitochondrial genes, effects on mitochondrial oxidative phosphorylation (OXPHOS) can be expected (Shedge et al., 2007; Arrieta-Montiel et al., 2009). The msh1×recA3 data set showed the strongest overlap in regulated transcript number with the other data sets (Table 2). This most probably reflects the fact that the msh1×recA3 mutant has several defects in mitochondrial physiology that were shared with the other experiments. However, 860 and 537 transcripts were specifically up- and down-regulated, respectively, compared with the
other experiments (Supplementary Table S1 at JXB online). These included four strongly up-regulated TFs: two AP2-EREBP TFs (At1g22810; RAP2.6, At1g43160), a myb-domain protein (MYB74, At4g05100), and a WRKY-TF (WRKY48, At5g49520), which were connected in a co-expression network together with 192 transcripts of the categories biosynthesis of secondary metabolites, plant–pathogen interactions, cysteine and methionine metabolism, phenylalanine metabolism, and phenylpropanoid biosynthesis, as well as 30 more TFs of various classes (Supplementary Fig. S1). The mshl×recA3 double mutant can be interpreted as an extreme case of mitochondrial dysfunction. In contrast to other experiments analysed here, drastic changes in growth, development, fertility, and stress resistance have been observed in this mutant (Shedge et al., 2007). Due to the loss of MSH1 and RecA3, many mitochondrial-encoded genes are affected in their expression simultaneously. As most of the mitochondrial-encoded genes are components of the OXPHOS complexes, the integrity of the ETC may be destabilized at different locations simultaneously rather than just a single protein or complex, that usually allows functional bypass. It is therefore not surprising that this mutant also shows the strongest transcriptomic reprogramming compared with the other experiments. The overlap in regulated transcripts with other experiments suggests simultaneous activation of several retrograde signalling pathways, only some of which are activated in the other individual experiments. Interestingly, the strongest transcriptomic overlap was observed with experiments in which complex I was absent or dysfunctional. The overlap in regulated transcripts with the strongest transcriptomic reprogramming compared with the other experiments. The overlap in regulated transcripts with other experiments suggests simultaneous activation of several retrograde signalling pathways, only some of which are activated in the other individual experiments. Interestingly, the strongest transcriptomic overlap was observed with experiments in which complex I was absent or dysfunctional. This provides an interesting correlation as complex I has the highest number of mitochondrial-encoded subunits of all ETC complexes (nine NAD genes in most plants including Arabidopsis; Rasmusson et al., 1998). Complex I is therefore expected to be particularly prone to misexpression as an outcome of mitochondrial genome rearrangements.

(vi) mtROS

One data set dealt with the loss of the mitochondrial superoxide dismutase (SOD) MSD1 in 3-week-old rosettes of Arabidopsis plants. An inducible RNAi approach was chosen to manipulate specifically mtROS levels (Schwarzlander et al., 2011) and to avoid pleiotropic effects that were observed in stable MSD1 antisense lines (Morgan et al., 2008). As a result of induction, MSD1 protein became undetectable by immunoblotting in rosettes after 12 d of dexamethasone treatment in two independent MSD1-inducible RNAi lines (msdl, lines 1 and 12) compared with the control line [induced line with an RNAi-hairpin specific for luciferase; pOpOff2(kan);:LUC (Wielopolska et al., 2005); data not shown; see Schwarzlander et al., 2011]. In Arabidopsis, MSD1 is the only known SOD in the mitochondrial matrix (Kliebenstein et al., 1998; Morgan et al., 2008). Therefore, loss of MSD1 can be expected to cause perturbed O$_2^-$ detoxification. This was reflected in slightly decreased aconitase activity, a marker for superoxide levels (Gardner, 2002), after 12 d of RNAi induction (to 88±4% and 81±3% of the control, respectively). However, no signs of general oxidative stress or acclimatization were detectable in the inducible msdl lines, including unchanged electrolyte leakage from plant roots, no changes in the total proteome (except for MSD1 itself), no changes in other SOD protein levels, and an unaffected mitochondrial glutathione redox state (unpublished data). Only 41 transcripts were changed in the msdl line (Supplementary Table S1 at JXB online). The transcripts which showed the strongest regulation coded for a pathogen-related protein (1.2-fold, PCC1, At3g22231), and QQS, a potential regulator of starch biosynthesis (~1.7-fold, QOS, At3g30720) (Li et al., 2009), respectively. Although only little is known about QQS function, similar repression has been demonstrated for a variety of biotic and abiotic conditions (Zimmermann et al., 2004). Eight and 11 transcripts were exclusively up- and down-regulated, respectively, in the msdl line and were not regulated in the other 10 data sets. Six of the up-regulated transcripts [including three wall-associated kinases (At1g21230, At1g21270, At1g22710), an FKBP-binding protein (At2g14560), and late up-regulated in response to Hyaloperonospora parasitica (LURP1, At5g48580)] were detected in the AttedII co-expression database. Only two transcripts were connected in a co-expression network with 24 more transcripts. The other four subnetworks were lying in close proximity to these (Supplementary Fig. S1). Several of the co-expressed transcripts included disease resistance proteins (TIR-NBS-LRR class), AAA-ATPase transcripts, and PCC1. Eight of the 11 down-regulated transcripts [including ethylene-responsive AP2-EREBP TF (At5g34310), a MYB-TF (transient testa 2, At5g35550), a transposable element gene (At2g11370), a maternal effect embryonic arrest protein (MEE38, At3g43160), and damaged DNA-binding protein (XRCC3, At5g37450)] did not share a connected co-expression network. However, it was obvious from the functional categories of the transcripts that like in other data sets analysed here genes of pathogen defences were affected in particular. Interestingly, no overlap between the msdl and AA data was observed (Table 2), which might hint at distinct mtROS signals produced in these experiments. The relatively mild transcriptomic response to the complete removal of MSD1 protein probably reflects the very specific stimulus of this system. Different from the other studies, the absence of MSD1 does not directly disrupt the OXPHOS machinery and is therefore less likely to cause major respiratory defects. In addition, superoxide is membrane impermeable, which limits the ROS stimulus to the matrix. It is therefore expected that the changes observed in this system are, albeit mild, particularly specific and biologically meaningful.

Marker transcripts for mitochondrial dysfunction

From the above comparisons, a list of marker transcripts that were regulated in response to a specific mitochondrial impairment was generated (Table 3). Transcripts that reflected a general mitochondrial stress response were selected from a comparison of mshl×recA3, the complex I mutants, as well as oligomycin and rotenone treatments, as the highest overlaps with the strongest log fold changes were observed between those experiments. Transcripts that
Table 3. Marker transcripts for mitochondrial dysfunctions (targets) extracted from the comparison of data sets by hierarchical clustering.
Data were processed as described in the Materials and methods. Expression values are given as fold ratios (log2). Blue and red indicate up- and down-regulation of transcripts, respectively.

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<th>ndufs1</th>
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<th>AA</th>
<th>aox1a</th>
<th>Oligomycin</th>
<th>AP9- uATP9</th>
<th>AP3- uATP9</th>
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were >1-fold (log2; either up- or down-) regulated in all these four experiments were searched. Ten transcripts that matched these criteria were detected: seven of these were up-regulated in all four experiments [RNase H domain-containing protein, At1g24090; NAC domain protein 13 transcription factor (ANAC13, At1g32870); embryo-abundant protein-related (At2g41380); CYP81D8 (At4g37370); unknown protein (At5g40690 and At2g41730); and protein phosphatase 2C (PP2C, At5g59220)], and one was down-regulated: tonoplast intrinsic protein 2:2 (TIP2:2, At4g17340) (Table 3). Those transcripts are considered general mitochondrial stress markers. The ‘embryo-abundant protein-related’ transcript was identified as a mitochondrial stress defence marker before, which could be confirmed here (Van Aken et al., 2009). Four of the seven up-regulated marker transcripts, including the TF ANAC13, were detected in a co-expression network together with AOX1a, the most commonly used marker transcript for mitochondrial dysfunction (Supplementary Fig. S1 at JXB online). ANAC13 and both unknown proteins are generally regulated by hydrogen peroxide (Inzé et al., 2011). PP2C was detected in a different co-expression network, and was reported to be highly induced by ABA treatment (Fujita et al., 2009). It functions specifically in SRK2D/E/I-mediated ABA signalling in response to water stress in the vegetative stage of Arabidopsis (Fujita et al., 2009). A general mitochondrial stress response appears, therefore, linked to ROS and ABA signalling.

Oxidative stress-related changes in gene expression

The AOX1a transcript, which is often used as a readout for mitochondrial retrograde signalling, was strongly >1-fold (log2] up-regulated in the msh1×recA3 mutant and upon rotenone and oligomycin treatment as well as to a lesser extent in the complex I mutants and upon AA treatment (0.20 to 0.5-fold) (Table 3). AOX1a transcript induction is known to be suppressed by antioxidants, suggesting that ROS play a direct role in signal transduction (Maxwell et al., 2002). Ho et al. (2008) identified 10 cis-acting regulatory elements (CAREs) in the promoter of the AOX1a gene. In a genome-wide search they identified 1141 genes that contained six or more of these CAREs. Most of these genes were regulated by oxidative, abiotic, and biotic stress (Ho et al., 2008). Those genes were searched in the analysed array data sets. A total of 908 were present in at least one of the data sets and 148–165 transcripts of CARE genes were regulated >0.5-fold (log2) upon rotenone treatment and in the complex I and msh1×recA3 mutants, respectively. Only 14 of these transcripts were regulated in aox1a; none of those was found to be regulated in the AA data sets, and only a minor number were regulated in the w-ATP9 and msd1 mutants (1–4 transcripts). This could hint at CARE gene regulation by a complex I-specific stimulus (complex I is likely to be impaired in msh1×recA3 too, see above). Alternatively, oxidative stress, which is known to impact on CARE genes, was absent in the latter experiments, which is also reflected in a less pronounced transcriptomic response. Oxidative stress signalling is induced by increased ROS generation that leads to oxidative damage of cellular components. It is therefore important to distinguish between oxidative stress signalling and redox signalling, which occurs under non-stress conditions. In principle, there are three types of redox signals: the first derives from the redox state of the quinone pools in the photosynthetic and mitochondrial electron transport chain, the second from thiol-containing redox-active compounds, such as thioredoxins, and the third from ROS themselves (Pfannschmidt, 2003; Apel, 2004; Dietz, 2008). Redox signals of all three categories can be regarded as important candidates in mitochondrial retrograde signalling (Dutilleul et al., 2003b). In this case, however, oxidative stress appeared to account for a significant proportion of the signalling stimuli that caused transcriptomic reprogramming in different systems of severe mitochondrial dysfuction.

Transcripts encoding ribosomal proteins and photosynthetic proteins are common targets of mitochondrial dysfunction

Individual marker transcripts are insufficient to get a picture of the actual functional output of mitochondrial signalling. Therefore, orchestrated transcript changes of functionally similar transcript groups were analysed across data sets. A functional class scoring algorithm with Hochberg correction was used, as implemented in the MapMan software (Usadel et al., 2005). Interestingly, the categories ‘protein synthesis’ (eight data sets), ‘photosynthetic light reactions’ (eight data sets), ‘protein targeting’ (seven data sets), ‘protein degradation’ (five data sets), ‘pentatricopeptide (PPR) repeat-containing proteins’ (six data sets), and ‘plasmamembrane intrinsic proteins (PIP7)’ (five data sets) were significantly regulated in the data sets (Supplementary Table S2 at JXB online). Importantly, although only minor fold changes were observed in the AA and msd1 microarrays, they showed a significant co-regulation of transcripts in the above-mentioned categories (Table 4). Although ~600–900 transcripts were regulated in the w-ATP9 lines, no significant functional categories were detected in the AP3-wATP9 line (hence it was not listed in Table 4), and only a few subcategories from protein synthesis, targeting, the cell wall, and the tricarboxylic acid (TCA) cycle were significantly regulated in the AP9-wATP9 line (Supplementary Table S2).

Although the functional category ‘biotic stress’ itself was not significantly regulated (except for msh1×recA3; Supplementary Table S2), a number of functional categories related to plant-pathogen interactions showed significant regulation in most experiments, including ‘redox’, ‘protein degradation’, ‘MYB-related transcription factors’ (five data sets each), ‘cell wall’, and ‘hormone metabolism’ (four data sets each) (Fig. S2; Supplementary Table S2 at JXB online). This, along with the high representation of individual regulated pathogen-related transcripts, points to plant–pathogen interaction as a general functional target of mitochondrial retrograde regulation.
Mitochondrial control of biotic stress responses is consistent with previous findings based on the study of marker genes (reviewed in Amirsadeghi et al., 2007). As mitochondrial electron transport is highly sensitive to external stimuli and controls mtROS release and cell death programmes (Scott and Logan 2008), the mitochondrion satisfies the requirements to act as an intracellular relay station of cellular pathogen defence. Candidate mechanisms for linking pathogen attack and mitochondrial impairment include ETC inhibition by SA (Xie and Chen, 1999). Cross-talk between ROS generation by NADPH oxidases at the plasma membrane and by the ETC in mitochondria in animal systems may suggest a similar connection in plants (Daiber, 2010). Mounting an appropriate defence response may therefore depend on retrograde signalling. In turn, genetic or pharmacological impairment of mitochondrial respiration may activate mitochondrially controlled pathogen-related gene expression via the same pathways.

For the functional category ‘protein synthesis’, changes for individual transcripts were mostly subtle but highly orchestrated for a large number of cytosolic, plastidic, and mitochondrial ribosomal proteins (Fig. 2). Interestingly, two groups of responses were revealed. While the transcripts coding for cytosolic ribosomal proteins were co-ordinately up-regulated in the ndufs4, ndufa1, msh1×recA1, msd1, and aox1a mutants, they were mostly down-regulated in the short-term inhibitor experiments (AA, rotenone, and oligomycin) (Fig. 2). This most probably reflects the fact that acclimation responses had occurred in the stable transgenic lines which were absent in the inhibitor treatments. This suggests that acute mitochondrial dysfunction leads to a repression of the cytosolic protein synthesis machinery, while chronic dysfunction triggers compensation and an induction. Transcripts encoding organellar ribosomal proteins responded differently from their cytosolic counterparts. In the complex I mutants (ndufa1 and ndufs4), inverse changes were observed for transcripts encoding mitochondrial (up-regulated) or plastidic ribosomal proteins (down-regulated), whereas in msh1×recA3 and aox1a mutants as well as upon oligomycin treatment, all transcripts of organellar ribosomal proteins were largely up-regulated (Fig. 2).

The transcripts coding for photosynthetic light reactions were also significantly regulated in eight data sets. While an orchestrated down-regulation was observed in seven data sets [msh1×recA3, ndufa1, ndufs4, rotenone, AA (WT), AA (pxcII F), and msd1] for photosystem I (PSI)- and PSII-encoding transcripts, a significant up-regulation was observed in the aox1a mutant under control conditions. This opposite response in a mutant with a defect in the alternative respiratory pathway strengthens once more the suggestion that retrograde control can be modulated by the route of electron flow. However, the down-regulation of transcripts encoding light reaction components is not necessarily dependent on the presence of the AOX protein, as a strong down-regulation of these transcripts was also observed in the aox1a mutant subjected to light and drought stress (Giraud et al., 2008), showing that there are other regulatory pathways controlling...
these transcripts under more severe stress. The expression of genes encoding photosynthetic and ribosomal proteins is, for example, also regulated by the cytosolic energy-sensing kinase SnRK1 which mediates general cellular starvation and sugar responses (Baena-González and Sheen, 2008; Smeekens et al., 2010). It will be interesting to see in the future whether a mitochondrial-derived retrograde signal also feeds into this pathway (Fig. 3).

A connection between organellar protein synthesis and retrograde signalling was also observed in Arabidopsis mutants that are defective in organellar protein biosynthesis due to down-regulation of the prolyl-tRNA synthetase gene (prors1, At5g52520) which is targeted to both chloroplast and mitochondria (Pesaresi et al., 2006). Only the combination of decreased translation rates in both organelles, due to defective prors1 expression, caused a down-regulation of photosynthesis-related transcripts (Pesaresi et al., 2006). However, a direct connection between the prors1 transcript level and photosynthetic gene expression was not observed in the present analysis. The msh1×recA3 mutant showed even a slightly increased prors1 transcript level while at the same time a strong repression of photosynthetic gene products was observed (Supplementary Table S2 at JXB online). In contrast, the aox1a mutant showed a strong and significant up-regulation of transcripts coding for photosynthetic proteins under control conditions, while transcripts coding for ribosomal proteins were strongly increased as in the msh1×recA3 mutant. This leads to two important conclusions: Changes in transcript levels encoding photosynthetic proteins (i) can be caused by mitochondrial-specific dysfunction and (ii) are not necessarily co-regulated with transcripts encoding proteins involved in organellar protein biosynthesis.

Experiments on Chlamydomonas reinhardtii have revealed a link between mitochondrial electron transport function and the expression of nuclear genes encoding photosynthetic
proteins (Matsuo and Obokata, 2006; Matsuo et al., 2011). It was found that active mitochondrial respiration induced photosynthetic transcripts and that this induction was blocked in mitochondrial respiratory mutants, as well as by the application of AA. Although the nature of the retrograde signal was not identified, it seems justified to consider mitochondrial electron transport an upstream stimulus for regulation of nuclear gene expression.

The findings presented here for Arabidopsis are in agreement with a general mitochondrial control of nuclear-encoded photosynthetic genes. It needs to be pointed out that all systems which rely on photosynthesis as their main energy source show this response consistently (eight out of 11 data sets). The remaining three data sets were gathered from overall heterotrophic systems, such as flowers and cultured cells. Complete consistency between systems provides strong evidence for mitochondrial control of photosynthesis on a transcriptional level. Although the effect has already been observed in individual systems (Dutilleul et al., 2003a; Garmier et al., 2008; Meyer et al., 2009), unambiguous interpretation has been difficult due to the possibility of a unique effect of the respective mitochondrial dysfunction or direct experimental impacts on plastidic status. For example, AA can also inhibit the cytochrome b6f complex in plastids (although treatments were performed in dark-adapted plants), and there is new evidence for dual targeting of MSH1 (but not RecA3) to mitochondria and plastids (Xu et al., 2011). The risk of inappropriate interpretation is minimized in this meta-analysis due to the consistent regulation of photosynthetic transcripts in all autotrophic systems analysed here, independent of the treatment or mitochondrial defect. It is unlikely that in all systems the plastid was directly affected (although indirect effects via altered mitochondrial status are a possibility). The present data therefore strongly suggest that mitochondrial status can determine the expression of nuclear-encoded photosynthetic transcripts. Such interorganellar cross-talk between both bioenergetic organelles via the nucleus complements the well-studied metabolic interaction between mitochondria and chloroplasts (Raghavendra and Padmasree, 2003). It could provide an important level of regulation to synchronize both organelles and guarantee efficient collaboration under changing conditions.

Conclusions and perspectives

Three main functional targets of mitochondrial retrograde signalling were identified: (i) protein synthesis; (ii) photosynthetic light reactions; and (iii) plant–pathogen interactions. Transcripts from these groups were affected by mild mitochondrial impairments as well as by severe mitochondrial and respiratory dysfunctions. Similar observations have been made in the individual studies already; however, this comprehensive meta-analysis reveals them as general and robust targets of mitochondrial signalling. Figure 4 summarizes a working model of retrograde signalling as discussed in this study. The key components of the retrograde signalling pathway(s) regulating the transcription factor ABI4 are still unknown and might include components of known ABA, ROS, and energy signalling pathways (Fig. 4).

The differential regulation of photosynthetic light reactions depending on the precise type of mitochondrial dysfunction...
reveals a new layer of complexity of mitochondria–chloroplast interaction and raises questions about the nature of signalling between them. Future research also needs to clarify the role of the coordinated transcript changes of ribosomal proteins which raises the possibility that mitochondrial retrograde control might not only influence the expression of genes at the promoter level, but could also act directly on the level of translation (Ho et al., 2008).

The outcomes of several forward genetic screens on mitochondrial retrograde signalling mutants are anticipated in the next years, and raise high hopes that the molecular components responsible for mitochondrial retrograde signalling in plants might be finally identified.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. Co-expression networks identified by Atted-II for transcripts which are solely regulated in the individual experiments.

Figure S2. MapMan display of regulated transcripts involved in the plant–pathogen response in individual experiments.

Table S1. Data table with log fold changes from all transcripts of the 11 analysed microarray experiments which were used in the meta-analysis, and lists of transcripts which were specifically regulated by a certain mitochondrial impairment.

Table S2. Functional class scoring results with Hochberg correction from individual experiments using the MapMan software. Functional bins with \( P \)-values are indicated.

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