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Inhibition of the electron transport strongly affects transcription and transcript levels in *Arabidopsis* mitochondria

Yan O. Zubo [a,1], Tatyana V. Potapova [a,b], Maria V. Yamburenko [a,1], Vladislav I. Tarasenko [b], Yuri M. Konstantinov [a,b,c], Thomas Börner [a,*]

[a] Institute of Biology-Genetics, Humboldt University, Chaussestr. 117, 10115 Berlin, Germany
[b] The Siberian Institute of Plant Physiology and Biochemistry SB RAS, Lermontova St., 132, Irkutsk 664033, Russia
[c] The Irkutsk State University, Sukhe-Batar St., 5, Irkutsk 664033, Russia

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ABSTRACT

Mitochondrial transcription rate and RNA steady-state levels were examined in shoots of *Arabidopsis* seedlings. The shoots were treated with inhibitors of complex III and IV of the cytochrome pathway (CP) and with an inhibitor of the alternative oxidase (AOX) of the mitochondrial electron transport chain. The inhibition of AOX and CP complexes III and IV affected transcription and transcript levels in different ways. CP and AOX inhibitors had opposite effects. Our data support the idea that the redox state of the electron transport chain is involved in the regulation of mitochondrial gene expression at transcriptional and post-transcriptional levels.

1. Introduction

The mitochondrial genomes of higher plants code for subunits of the respiratory chain complexes I to V, proteins involved in cytochrome c biogenesis, a subunit of the twin arginine translocase (protein transport), and components of the translation machinery, i.e., tRNAs, rRNAs and ribosomal proteins. Most of the proteins needed for respiration, mitochondrial gene expression and all other mitochondrial functions are encoded in the nuclear genome (Knoop et al., 2011; Unseld et al., 1997). Since the information of two genomes is required for the biogenesis and function of complexes I to V of the mitochondrial electron transport chain (ETC.) and of the mitochondrial ribosomes, and since mitochondrial respiration is central to cellular metabolism, it can be expected that regulatory mechanisms coordinate the expression of genes in the nucleus and mitochondria. There is indeed a growing body of evidence that retrograde signaling from mitochondria to the nucleus regulates the transcription of nuclear genes in response to the functional state of the ETC. components (Rhoads, 2011; Schwarzländer and Finkemeier, 2013; Schwarzländer et al., 2012; Van Aken and Whelan, 2012; Welchen et al., 2014).

Plant mitochondria differ from animal mitochondria in that they have a cyanide-resistant alternative oxidase (AOX) and alternative NAD(P)H dehydrogenases and thus are capable of non-phosphorylating respiration (Millar et al., 2011). Unlike the terminal oxidase of the cytochrome pathway (CP), AOX is a non-energy conserving oxidase encoded by a small gene family (Clifton et al., 2006; Millar et al., 2011). The ability to normalize the redox state and to protect the cell against photo-induced damage during photosynthesis makes AOX important for chloroplast–mitochondria interrelationships (Yoshida et al., 2011). Stress conditions (drought, low temperature, high light, high salt and wounding) induce AOX expression, and the enzyme is considered crucial for the stress response of plants (Hiser and McIntosh, 1990; Vanlerberghe, 2013). Both AOX and CP are involved in retrograde regulation of nuclear gene expression under stress and non-stress conditions. Perturbations in the function of mitochondrial ETC. alter the expression of many nuclear genes including genes for the energy metabolism and protein synthesis in mitochondria (Garmier et al., 2008; Giraud et al., 2009; Millar et al., 2011; Saisho et al., 2001; Tarasenko et al., 2009; Van Aken and Whelan, 2012; Watanabe et al., 2008; Welchen et al., 2014; Zarkovic et al., 2005).

In stark contrast to the situation with regard to nuclear transcripts, little is known about the effects of disturbed ETC. function on mitochondrial gene expression in plants. Mitochondrial RNA accumulation was tested in a number of mutants with defective complex I or complex III of the ETC. (Kühn et al., 2009, 2011; Liu et al., 2010b; Meyer et al., 2009; Sosso et al., 2012; Sung et al., 2010; Wang et al., 2012). All these studies revealed positive effects on mitochondrial gene expression. However, these studies reflect the effects of long-term functional

Abbreviations: ETC, electron transport chain; CP, cytochrome pathway; AOX, alternative oxidase; MTR, mitochondrial transcription rate; KCN, potassium cyanide; AA, antimycin A: SHAM, salicylhydroxamic acid; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

* Corresponding author. Tel.: +49 3020938142.
E-mail address: thomas.boerner@rz.hu-berlin.de (T. Börner).
[1] Current address: Dartmouth College, Department of Biological Sciences, 78 College St., Hanover, NH 03755, USA.

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deficiencies and cannot reveal potential changes in gene expression due to short-term alterations in the redox state of the ETC. and of respiration in general. There is a report on activating and the repressive effects of several ETC. inhibitors on the incorporation of tritium labeled UTP into acid-insoluble, RNase-sensitive material in isolated potato mitochondria. The results suggest a redox control of RNA synthesis in potato mitochondria but individual genes have not been studied (Wilson et al., 1996).

In the present work we developed an experimental model in which shoots of 12-day-old *Arabidopsis* seedlings were treated with inhibitors targeting AOX and the CP complexes III and IV. They were subsequently subjected to comprehensive analyses of RNA synthesis and transcript abundance in mitochondria. Our data suggests the regulatory roles of both CP and AOX in mitochondrial gene expression at the levels of transcription and transcript accumulation.

2. Material and methods

2.1. Plant material and treatment

Seedlings of *Arabidopsis thaliana* (ecotype Col-0) were grown in soil under long-day conditions (16 h photoperiod; light intensity 130 μmol m^{-2} s^{-1}) at 23 °C. Shoots of 12-day-old seedlings (counting from the day of sowing) were harvested and floated in inhibitor solutions or control solution for 4 h under continuous illumination (white light, 130 μmol m^{-2} s^{-1}). The final concentration of the inhibitors was: 1 mM potassium cyanide (KCN), 10 μM antimycin A (AA), 1 mM salicylhydroxamic acid (SHAM)), and 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). EtOH was used to solubilize SHAM and AA; the EtOH concentration in both mock and experimental treatment solutions was 0.2%.

2.2. Isolation of mitochondria

Mitochondria were isolated by differential centrifugation followed by purification in a discontinuous Percoll gradient as described by Kühn et al. (2009) with some modifications. Seedling shoots (10 g) were ground in 90 mL of extraction buffer composed of 0.3 M sucrose, 5 mM anhydrous sodium pyrophosphate, 2 mM EDTA, pH 7.5, 1% (*w*/*v*) PVP40, 1% (*w*/*v*) BSA, 5 mM cysteine and 5 mM 2-mercaptoethanol. The homogenate was filtered through 4 layers of Miracloth and centrifuged for 15 min at 5000 *g*. The supernatant was recovered and centrifuged for 20 min at 22,000 *g*. The pellet was resuspended in 40 mL of washing buffer (50 mM Tris–HCl, pH 7.0, 10 mM $MgCl_2$, 10 mM KCl and 4 mM 2-mercaptoethanol) and then centrifuged for 5 min at 3000 *g*. The pellet was discarded and the supernatant centrifuged for 15 min at 18,000 *g*. The pellet was resuspended in 8 mL of washing buffer and homogenized with a glass potter. The suspension was centrifuged for 5 min at 3000 *g* and the supernatant was loaded onto an 18%, 25%, and 50% Percoll step gradient (in washing buffer) and centrifuged for 30 min at 40,000 *g*. The fraction of intact mitochondria between the 25% and 50% Percoll layers was collected and washed 3 times with 20 mL of washing buffer followed by centrifugation for 10 min at 22,000 *g*. Finally, the pellet was resuspended in 1 mL of washing buffer. Mitochondria equivalent to 200 mg of mitochondrial protein were used in all run-on transcription assays. 50 μL of the suspension was used to determine protein concentration with a Bio-Rad Protein Assay Kit (Bio-Rad). All steps were performed at 4 °C.

2.3. Measurement of O_2 uptake

The O_2 uptake rate by shoots of 12-day-old *A. thaliana* seedlings exposed for 4 h to the respiratory inhibitors was measured using a Clark-type thermoelectrically controlled oxygen electrode (*Oxytherm System, Hansatech Inst*., Pentney, Norfolk, UK) at 25 °C. The shoots were cut into small pieces (1.0–1.5 mm^2 each) with a razor blade. Measurements were carried out after suspending the small shoot segments (70–80 mg in total) in 1.5 mL of a air-saturated solution containing sucrose (100 mM), HEPES (50 mM), MES (10 mM, pH 6.6), $CaCl_2$ (0.2 mM) according to Noguchi et al. (2001).

2.4. Probe design and run-on transcription assay

DNA probes for 34 mitochondrial genes were obtained by PCR using gene-specific primers (Supplementary Table S1). Gene-specific probes were denatured and dot-blotted onto Nylon Hybond-N + membranes (Amersham Pharmacia Biotech, UK) as described (Zubo et al., 2011). Run-on transcription assays were performed according to published protocols (Giegé et al., 2000; Kühn et al., 2009) with the following modifications. Intact mitochondria were pelleted and resuspended in 100 μL of transcription buffer containing 20 mM Tris, pH 7.6, 40 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 0.5 mM each of ATP, GTP, and CTP, 0.01 mM UTP and 40 units of ribonuclease inhibitor (Invitrogen, Germany) at 4 °C. Transcription was started by adding 50 μCi [α-^{32}P]UTP (Amersham, UK) and placing tubes at 25 °C. Reactions were stopped after 15 min by adding 100 μL of stop buffer (50 mM Tris–HCl, pH 8.0, 25 mM EDTA, 5% sarkosyl). ^{32}P-labelled transcripts were isolated by phenol/chloroform extraction and EtOH precipitation. Hybridization of transcripts with dot blotted DNA probes was performed as described (Zubo et al., 2011). Radioactive signals were detected and quantified by scanning using the Molecular Imager FX and Quantity One software (Bio-Rad, USA). Five genes (*nad6*, *ccb382*, *trnN*, *matR*, *orf153a*) were excluded from the quantifications because their signals were below the level of detection or due to significant variability between biological replicates. The common logarithms of the inhibitor/control ratios were calculated; the common logarithms +0.3 and −0.3 correspond to 2-fold up- and down-regulation, respectively. Figs. 2D, E, F, and G present means ± SD calculated from three independent biological repetitions of the experiments. The effect of inhibitor treatment on transcription was considered significant if the signals differed at least 2-fold from the control treatment.

The degree of cross-hybridization was tested experimentally to validate the method. Membranes with mitochondrial DNA probes were hybridized with transcripts obtained from chloroplast run-on reactions (Zubo et al., 2011). After hybridization and washing no signals of radiolabeled chloroplast transcripts were detected (data not shown) indicating a high level of specificity under the experimental conditions employed.

2.5. RNA isolation, RNA blot hybridization, and quantitative real-time PCR

Total RNA isolation and blot hybridization were performed as described in Yamburenko et al. (2013).

The *atp1*, *atp9*, *trnS* and *trnY* transcripts were tested by hybridization with single-stranded radioactive probes that were generated by PCR with single reverse primers (Supplementary Table S1) using double-stranded fragments as template. The reaction was performed in the presence of [α-^{32}P]-dCTP (Amersham, UK). The experiments were repeated twice with independently isolated RNAs; transcript levels are expressed as percent of the control treatment with water.

The transcript levels of the nuclear *AOX1a, MSM1,* and *DNAJ* genes were determined by quantitative real-time PCR (qRT-PCR). We synthesized cDNA using oligo$(dT)_{12-18}$ and M-MuLV Reverse transcriptase (Fermentas, Lithuania). qRT-PCR was performed with the SYBR Select Master Mix (Applied Biosystems, USA) and the CFX96 Real-Time PCR Detection System (Bio-Rad, USA). All reactions were performed in triplicate. The expression of each gene was normalized to the expression of *YLS8* (at5g08290) (Hong et al., 2010). qRT-PCR analyses were repeated four times with independently isolated RNA samples.