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Regulation of alternative oxidase 1 in *Chlamydomonas reinhardtii* during sulfur starvation

Zhanneta Zalutskaya, Valentina Filina, Mariya Ostroukhova, Elena Ermilova*

Biological Faculty, Saint-Petersburg State University, Universitetskaya nab. 7/9, Saint-Petersburg, 199034, Russia

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Abstract

The mitochondrial respiratory chain in plants, some protists and many fungi consists of the ATP-coupling cyanide-sensitive cytochrome pathway and the cyanide-resistant alternative respiratory pathway. The alternative pathway is mediated by alternative oxidase (AOX). Although AOX has been proposed to play essential roles in nutrient stress tolerance of plants and protists, the effects of sulfur (S) deprivation, on AOX are largely unknown. The unicellular green alga *Chlamydomonas reinhardtii* reacts to S limitation conditions with the induced expression of many genes. In this work, we demonstrated that exposure of *C. reinhardtii* to S deprivation results in the up-regulation of *AOX1* expression and an increased AOX1 protein. Furthermore, S-deprived *C. reinhardtii* cells display the enhanced AOX1 capacity. Moreover, nitrate assimilation regulatory protein (NIT2) is involved in the control of the *AOX1* gene expression in the absence of S. Together, the results clearly indicate that AOX1 relates to S limitation stress responses and is regulated in a NIT2-dependent manner, probably together with yet-unknown regulatory factor(s).

Keywords: Alternative oxidase 1; Artificial microRNA approach; *Chlamydomonas reinhardtii*; Sulfur deprivation; Quantitative real-time RT-PCR

Introduction

The mitochondrial respiratory chain in plants, some protists and many fungi consists of the ATP-coupling cyanide-sensitive cytochrome pathway and the cyanide-resistant alternative respiratory pathway (Finnegan et al. 2004; Millar et al. 2011). The alternative pathway is not coupled to ATP synthesis and mediated by alternative oxidase (AOX). As AOX bypasses proton pumping complexes III and IV of the cytochrome pathway, it reduces ATP generation and the energy yield of respiration (Affourtit et al. 2002; Sluse and Jarmuszkiewicz 1998).

AOX is a membrane-bound ubiquinol oxidase that catalyzes oxidation of ubiquinol and the reduction of oxygen to water. As described earlier AOX proteins are encoded by two subfamilies of genes *AOX1* and *AOX2* (Affourtit et al. 2002). In *Chlamydomonas reinhardtii*, AOX are monomeric fungi-type proteins that are encoded by two genes of discrete subfamilies, *AOX1* and *AOX2* (Baurain et al. 2003; Dinant et al. 2001). It has been shown that in higher plants, the *AOX* genes are induced in response to various stimulants (Van Aken et al. 2009; Vanlerberghe et al. 2009). In *C. reinhardtii* both the *AOX1* gene (Molen et al. 2006; Zalutskaya et al. 2015, 2016) and the *AOX2* gene (Ostroukhova et al. 2017) are significantly expressed upon stress conditions. One of the important stress is the dysfunction in mitochondrial electron transport chain complexes I, III and IV (Juszczuk et al. 2012; Vanlerberghe and McIntosh 1994). Thus any stimulus

Abbreviations: AOX, alternative oxidase; amiRNA, artificial microRNA; qRT-PCR, quantitative real-time PCR.

*Corresponding author.
E-mail address: e.ermilova@spbu.ru (E. Ermilova).

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resulting in cytochrome pathway complex dysfunction will lead to induction of AOX.

Sulfur (S) is an essential element that plays important roles in many metabolic processes. S can be limiting in the environment and strongly influence ecosystem composition (Scherer 2009). Algae respond to S deficiency by increasing their capacity for scavenging S from both internal and external resources (Giordano and Raven 2014; Pollock et al. 2005; Zhang et al. 2004). Interestingly, that in *C. reinhartii*, the *AOX1* gene is found in a gene cluster group involved in the nitrate assimilation pathway (Quesada et al. 1993). These cluster genes are under the control of the nitrate-specific regulatory transcription factor NIT2 (Camargo et al. 2007; Fernández et al. 1989; Quesada et al. 1998). It has been evidenced that *AOX1* is induced following nitrogen (N) starvation (Park et al. 2015) or by a change in nitrogen source from ammonium to nitrate (Molen et al. 2006). To understand the roles that AOX1 plays in defending against such metabolic fluctuations, we need to determine nutrient stresses leading to changes in AOX gene expression and AOX protein abundance.

In this work, we focused on *AOX1* transcriptional responses triggered by S starvation and compared them to corresponding changes in protein abundance and AOX1 capacity. The results indicate that in S-deprived *C. reinhardtii* cells the *AOX1* gene expression is regulated in a NIT2-dependent manner.

Material and Methods

Strains and growth conditions

The strains cw15-325 (cw15*mt*$^{+}$*arg7-8*, used as WT) and CC124 (*NIA1 NIT2* mt^{-}) was kindly provided by Dr. M. Schroda (University of Kaiserslautern, Germany). The strain nit2 (cw15 arg7 + *NIA1:ARS* mt + *Rbc:APHVIII NIT2*$^{-}$) were obtained from Dr. E. Fernández (University of Córdoba, Spain). Cells were grown mixotrophically in Tris-acetate-phosphate (TAP) medium (Gorman and Levine 1965) under continuous illumination with white light (fluence rate of 45 $\mu E\, m^{-2}\, s^{-1}$) at 22 °C. The TAP medium was supplemented with 100 mg/L of arginine when required. To induce sulfur deprivation of the strains used, the cells grown in TAP medium in the light were washed twice with sulfur-free medium (TAP-S), resuspended in the TAP-S medium and subsequently divided in two samples, one sample was incubated in the dark and the other was incubated under light for different periods. TAP-S medium was prepared as reported previously (Davies et al. 1994).

Protein and chlorophyll content

Cell culture samples (1–2 10^6 cells/mL in 10 mL) were harvested by centrifugation, the supernatant was discarded, and the pellet was resuspended in 0.1 M DTT, 0.1 M Na_2CO_3. Protein content was determined using the method described by Popov et al. (1975). Chlorophyll was extracted from whole cells with acetone. 0.6 mL of culture was centrifuged and the pellet resuspended in 83% acetone to extract pigments. Cellular debris was pelleted by centrifugation and chlorophyll a and b levels were determined spectrophotometrically (SmartSpec Plus, BioRad) in the supernatant, by measuring optical absorbance at 652 nm. Calculations of total chlorophyll (μg/mL) were performed as previously described (Harris 2008).

Synthesis of cDNA and quantitative real-time PCR (qRT-PCR) analysis

Total RNA was isolated as described previously (Ermilova et al. 2010). The quality of the RNA preparations was estimated by agarose gel electrophoresis, and RNA concentration and purity were determined spectrophotometrically (SmartSpec Plus, Bio-Rad). Reverse transcription was performed with RevertAid H Minus First Strand cDNA Synthesis Kit according to the manufacturerís instructions (Thermo Scientific). Primers for RT-qPCRs were chosen based on ≥90% primer efficiency and on a single melt curve. The primer pairs used for qRT-PCR were 5′-ACTTGGATCTGTGTGCCTGG-3′ and 5′-CTGGGCCGGTATCTACGTTC-3′ for *AOX1*, 5′-GAGTTTCAGGACCCGGATGG-3′ and 5′-AATAAACCGCAACCCCAGGT-3′ for *AOX2*, and 5′-CTTCTCGCCCATGACCAC-3′ and 5′-CCCACCAGGTTGTTCTTCAG-3′ for *RACK1*. Each reaction contained the master mix, 5% DMSO, 200 nM of each primer, and cDNA corresponding to 10 ng input RNA in the reverse transcriptase reaction. Real time qPCR was performed on the Light Cycler Instrument (CFX96 Real-Time PCR Detection System, Bio Rad) using SYBR Green I as a fluorescent dye. The reaction conditions were as follows: 95 °C for 3 min, followed by cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 60 s up to a total of 40 cycles. Controls without template or reverse transcriptase were included. The threshold cycle (Ct), defined as the fractional cycle at which the fluorescence signal becomes significantly different from the baseline was manually determined by the fit-point method provided by the LightCycler software. Gene expression ratios were normalized to *RACK1* using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Sulfur starvation treatments had no effect on the accumulation of *RACK1* transcripts in *Chlamydomonas* cells (Minaeva et al. 2017). Two or three biological replicates were averaged for each sample and three technical replicates were estimated in each biological experiment. Student's *t*-tests were used for statistical comparisons. P-values of <0.05 were considered as significant.

Generation of AOX2 knock-down strain

The miRNA targeting *C. reinhardtii AOX2* was designed as described (Molnar et al., 2009) using theWMD3 tool at