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The *Chlamydomonas* alternative oxidase 1 is regulated by cadmium stress: New insights into control of expression

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ABSTRACT

Although mitochondrial alternative oxidase (AOX) has been proposed to play essential roles in stress tolerance of plants, the effects of cadmium (Cd), an important environmental pollutant, on AOX are largely unknown. The green alga *Chlamydomonas reinhardtii* reacts to Cd stress conditions with the induced expression of many genes. In this work, we demonstrated a sharp increase in AOX1 transcript and protein abundance under Cd stress. Furthermore, *C. reinhardtii* cells displayed a large increase in alternative respiration in response to Cd treatment. Cd-induced AOX1 production and alternative pathway capacity were lower in illuminated cells than in dark-adapted cells. Moreover, in dark-adapted unstressed cells, AOX1 was significantly expressed. The results clearly indicate that the dark-mediated signals function in an additive manner with Cd-mediated signals, and thus likely act in parallel pathways to regulate the expression of *AOX1*.

1. Introduction

Cadmium (Cd) present at high concentrations in industrial areas (Bhagure and Mirgane, 2011; D'Emilio et al., 2013) is one of the most toxic heavy metals for living organisms (Nagajyoti et al., 2010; Thévenod, 2009). Contamination of freshwater and soils with Cd often has pronounced negative effect on plants, which can serve as entrance pathways into other organisms in the food chain (Gallego et al., 2012; Clemens et al., 2013). A better understanding of Cd-induced cellular and physiological responses in plants will therefore contribute to the development of strategies for prevention of Cd toxicity for human health.

Cd toxicity is a complicated phenomenon, related to disorder of metabolism in different cell compartments. Cd exposure might then lead to damage to proteins, lipids and nucleic acids (Watanabe et al., 2003). The main route of toxicity for this metal is through the binding to sulfhydryl, carboxyl and imidazole groups of proteins, causing inhibition of many physiological processes, including photosynthesis and respiration (Prasad et al., 1998; Aravind and Prasad, 2004; Wang and Wang, 2008). This, in turn, can be the reason for enhanced reactive oxygen species (ROS) formation leading to oxidative stress (Cuypers et al., 2012).

Chloroplasts are more intensively studied in the light of photosynthesis and its accompanying ROS production under metal stress conditions (Kučera et al., 2008; Vega et al., 2006). However, research has extended to plant mitochondria in recent years (Sharma and Dietz, 2009; Noctor et al., 2007). Some of these works suggested that plant mitochondria might be important targets of Cd toxicity (Heyno et al., 2008; Bi et al., 2009; Verbruggen et al., 2009). Exposure to Cd impairs proper mitochondrial functioning partly by affecting the organellar redox balance (Smiri et al., 2010). Unlike animal, the plant mitochondria possess unique components as alternative terminal oxidase (AOX) and alternative NAD(P)H dehydrogenases (Noctor et al., 2007; Sweetlove et al., 2007; Dutilleul et al., 2003).

AOX mediates cyanide-resistant respiration, which bypasses proton-pumping complexes III and IV of the cytochrome pathway to directly transfer electrons from reduced ubiquinone to molecular oxygen (Finnegan et al., 2004). AOX is currently considered as a regulator of stress responses in plants (Rhoads et al., 2006; Van Aken et al., 2009). As analysis of the AOX respiration under Cd treatment in the green lineage has yet only been performed on photosynthetic organism *Euglena gracilis* (Castro-Guerrero et al., 2008), in barley plants (Garmash and Golovko, 2009) and in *Arabidopsis thaliana* (Keunen et al., 2015, 2016), we have carried out a study on the green alga *Chlamydomonas reinhardtii* (herein referred to as *Chlamydomonas*). To study

Abbreviations: AOX1, alternative oxidase 1; COX3, subunit III of cytochrome c oxidase; MnSOD3, manganese-containing superoxide dismutase 3; qRT-PCR, quantitative real-time PCR.

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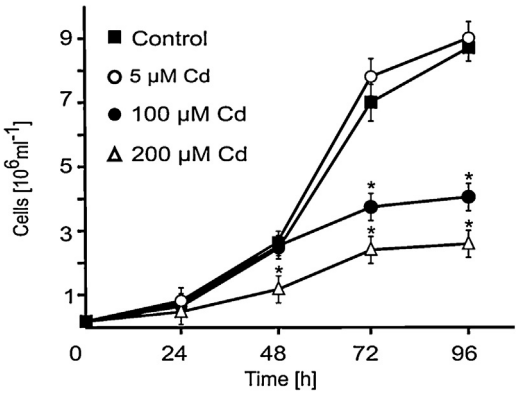

Fig. 1. Growth of the strain cw15-325 upon exposure to Cd.
Cells were grown in TAP medium containing 7.5 mM ammonium and 1 g acetate. Cell number was analyzed at the indicated times for cultures as they grew continuously in the light with or without $CdCl_2$ in the medium. Each data point represents the average of three independent experiments (±SE). Significant differences with respect to the control are indicated with *.

fundamental problems in plant mitochondria, *Chlamydomonas* has proved an excellent model organism (Rochaix et al., 1998; Harris, 2009). Moreover, it has the potential to be an ideal model system to study stress responses because cells can be grown under highly defined conditions and stress can be applied homogeneously to all cells in a culture (Zalutskaya et al., 2015; Schroda et al., 2015).

In *Chlamydomonas*, AOX is a monomeric fungi-type protein that is encoded by two genes, *AOX1* and *AOX2* (Dinant et al., 2001; Baurain et al., 2003). It has also been shown that only the *AOX1* gene is significantly expressed (Molen et al., 2006; Ermilova et al., 2010). This gene is found in a gene cluster group involved in the nitrate assimilation pathway (Quesada et al., 1993). It has been evidenced that, two distinct pathways regulate *AOX1* transcription in *C. reinhardtii*: one in response to a metabolic shift brought about by a change in nitrogen source from ammonium to nitrate and a second in response to oxidative stress (Molen et al., 2006).

Like other stresses Cd treatment is sensed by *C. reinhardtii* and elicits a coordinated cell response (Nagel et al., 1996; Gillet et al., 2006; Vega et al., 2006). Although many aspects of how *Chlamydomonas* responds to Cd stress have been studied (Aksmann et al., 2014; Nowicka et al., 2016), many questions remain unanswered. One of them is whether alternative respiration responds to Cd exposure. Another open question is related to how AOX abundance is controlled under Cd stress conditions. In this study, we investigated the regulation of *Chlamydomonas* AOX1 in response to Cd stress.

2. Material and methods

2.1. Strains and growth conditions

The strain cw15-325 (cw15*mt*$^+$*arg7-8*) was kindly provided by Dr. M. Schroda (University of Kaiserslautern, Germany). Cells were grown mixotrophically in Tris-acetate-phosphate (TAP) medium (Gorman and Levine, 1965) under continuous irradiation with white light (fluence rate of 45 $\mu E\,m^{-2}\,s^{-1}$) at 22 °C. The TAP medium was supplemented with 50 $mg\,L^{-1}$ of arginine. The number of cells was recorded microscopically with use of a counting chamber.

2.2. Chemical treatments

Based on a preliminary growth inhibition test with a range of $CdCl_2$ concentrations (Fig. 1), 100 μM $CdCl_2$ was selected as exposure final concentration. For experiments, cells grown at 22 °C to log phase ($1–2 \times 10^6$ cells mL^{-1}) were collected by centrifugation, resuspended in culture medium with 100 μM $CdCl_2$ and subsequently divided in two samples, one sample was incubated in the dark and the other was incubated under light for different periods. RNA and protein samples were prepared from cells collected at 0.25, 0.5, 1, 2, 3, 4, 6, 24 or 48 h after Cd exposure. For the peroxide treatment experiment, aliquots of cells were taken at 1, 3 and 5 h after addition to cultures of 1 mM H_2O_2. Control cultures were incubated without Cd or H_2O_2. At each harvesting time the number of cells was measured employing a counting chamber and the viable cells were estimated microscopically with use of 0.05% (v/v) Evans blue (DIA-M, Russia) as described (Baker and Mock, 1994). Stained (non-viable) and unstained (viable) cells were observed and counted. 400 cells from each sample were examined for three biological replicates.

2.3. 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). RNA (1 μg) was treated with RNase-Free DNase I (Fermentas) in 10 μL at 37 °C for 15 min. The reaction was stopped with 0.43 μL of 50 mM EDTA at 80 °C for 10 min.

Reverse transcription was performed with RevertAid H Minus First Strand cDNA Synthesis Kit according to the manufacturer's instructions (Thermo Scientific). Primers for RT-qPCRs were chosen based on ≥90% primer efficiency and on a single melt curve. They are listed in Table 1. 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. All reactions were performed in triplicate with at least two biological replicates. 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

Table 1
List of the employed primers.

Target gene	Primer name	Sequence (5′–3′)	Amplicon size (bp)
AOX1	*AOX1F*	ACTTGGATCTGTGTGCCTGG	82
	AOX1R	CTGGGCCGGTATCTACGTTC	
MSD3	*MSD3F*	GGAGATTGTCAGCCAGATCG	145
	MSD3R	GGAGGAGTTGAAGGTGGTCA	
COX3	*COX3F*	CCTGGAGTACAACGAGACACCC	191
	COX3R	ACGAAATGCCAGTACAGGATGG	
RACK1	*RACK1F*	CTTCTCGCCCATGACCAC	104
	RACK1R	CCCACCAGGTTGTTCTTCAG	