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# Functional expression of plant alternative oxidase decreases antimycin A-induced reactive oxygen species production in human cells

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ABSTRACT

**Alternative oxidase (AOX) plays a pivotal role in cyanide-resistance respiration in the mitochondria of plants, fungi and some protists. Here we show that AOX from thermogenic skunk cabbage successfully conferred cyanide resistance to human cells. In galactose medium, HeLa cells with mitochondria-targeted AOX proteins were found to have significantly less reactive oxygen species production in response to antimycin-A exposure, a specific inhibitor of respiratory complex III. These results suggest that skunk cabbage AOX can be used to create an alternative respiration pathway, which might be important for therapy against various mitochondrial diseases.**


## 1. Introduction

Alternative oxidase (AOX) is a mitochondrial enzyme that plays a central role in cyanide-insensitive respiration in eukaryotic taxa excluding mammals [1]. AOX acts as an alternative terminal oxidase in the electron transfer chain (ETC). It catalyzes the oxidation of ubiquinol, reducing molecular oxygen to water. Therefore, it is believed that as a result of bypassing two proton translocation sites in the cytochrome respiratory pathway (complexes III and IV), the redox energy which is not conserved for ATP synthesis is released as heat [2]. In fact, the massive respiratory burst that occurs during anthesis in thermogenic plants, such as the arum lily family (Araceae), has been attributed to the up-regulation of a cyanide-resistant alternative respiratory pathway [3–5]. Separate from thermogenic plants, previous studies in non-thermogenic plants have shown that the presence of AOX can prevent the prolonged reduction of ubiquinone, a status that would otherwise lead to reactive oxygen species (ROS) production, while allowing continuous operation of the ETC and the tricarboxylic acid cycle (TCA) [6].

In many organisms, ROS participate in cell signaling or injury and most of the intracellularly produced ROS originates in the mitochondrial ETC, in particular from complexes I and III [7,8]. In humans, uncontrolled increases in cellular ROS levels are thought to contribute to a wide range of pathological conditions, including aging, cancer, metabolic syndrome, and neurodegenerative and mitochondrial diseases [9]. Consequently, the quenching of excessive ROS should be one of the primary goals of therapies aimed at relieving the harmful consequences of pathological respiratory chain deficiency.

It has been shown that human cells can exhibit cyanide-resistant respiration through the allotopic expression of an animal *AOX* gene from ascidian *Ciona intestinalis* [10]. It has also been shown that AOX expression abrogated the apparent induction of superoxide dismutase (SOD) activity caused by antimycin A (AA), a mitochondrial complex III inhibitor [10]. Nevertheless, it is not clear whether mitochondrial AOX is capable of preventing the increase in ROS production provoked by AA in human cells.

In the present study, we show that plant AOX from the thermogenic skunk cabbage *Symplocarpus renifolius* [11] can be

*Abbreviations:* AOX, alternative oxidase; AA, antimycin A; BSA, bovine serum albumin; CCCP, carbonyl cyanide m-chlorophenyl hydrazone; CM-$H_2$DCFDA, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester; COX, cytochrome *c* oxidase; ETC, electron transfer chain; $H_2$DCF, 2′,7′-dichlorofluorescein; GC/MS, gas chromatography/mass spectrometry; MTS, mitochondrial targeting signal; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SOD, superoxide dismutase; TCA, tricarboxylic acid cycle

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functionally expressed in the HeLa cell line and that AOX expression lowers AA-initiated ROS production. Interestingly, this AOX protection against mitochondrial oxidative stress was observed in cells grown in galactose medium with active respiration. Our findings should be helpful for future investigation of dysfunctions in cytochrome respiration underlying various mitochondrial diseases in human cells.

## 2. Materials and methods

### 2.1. Subcellular localization and Western blotting

Subcellular localization of transfected gene product was determined using a Subcellular Proteome Extraction Kit (Calbiochem Inc., San Diego, CA, USA). Twenty-four hours after transfection (Supplementary data), HeLa cells were trypsinized and fractionated according to the manufacturer's protocol. The protein concentration was measured using the Bradford method (Bio-Rad Laboratories Inc., Hercules, CA, USA) with bovine serum albumin (BSA) as a standard. Each sample (20 μg) was fractionated by SDS–PAGE on a 12.5% acrylamide gel and transferred onto PVDF membranes (Immobilon-P; Millipore Inc., Bedford, MA, USA) using a semi-dry blotting system (HorizBlot; Atto Inc., Tokyo, Japan). Prestained protein markers (11–170 kDa; Fermentas Inc., Burlington, Ontario, Canada) were used for molecular mass estimation. The membranes were incubated for 1 h at room temperature in 5% (w/v) skim milk in Tris-buffered saline (137 mM NaCl, 2.68 mM KCl, and 25 mM Tris/HCl, pH7.4) containing 0.1% (v/v) Tween 20 (TBS-T), and then overnight at 4 °C in TBS-T with primary antibodies against AOX (mouse monoclonal antibody generated from *Sauromatum guttatum* AOX [12]), μ-calpain (mouse monoclonal antibody; Sigma–Aldrich Inc., St. Louis, MO, USA), and Complex III core 2 subunit (CIII; mouse monoclonal antibody; Invitrogen Inc., Carlsbad, CA, USA). Detection was achieved according to our previously reported method [11].

### 2.2. Measurement of oxygen consumption

Cells were trypsinized and the number of living cells was determined using a trypan blue dye exclusion assay [13]. Endogenous whole cell respiration was measured using a Clark-type electrode (Oxy1, Hansatech Instrument Inc., King's Lynn, Norfolk, UK) at 37 °C in individual growth media (Supplementary data). The oxygen concentration in air-saturated water at 37 °C was estimated to be 200 μM in each experiment.

### 2.3. Measurement of ROS production by flow cytometry

Intracellular ROS generation was detected by means of an oxidation-sensitive fluorescent probe dye [5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-$H_2$DCFDA)] (Invitrogen), as a cell-permeable indicator of ROS and in particular $H_2O_2$. This fluorescent probe dye allows for covalent attachment to intracellular components once it is deacetylated intracellularly by a non-esterase, permitting longer retention within the cell. The deacetylated $H_2$DCF (2′,7′-dichlorofluorescein) is non-fluorescent until oxidation occurs within the cell. Twenty-four hours after transfection, cells were trypsinized and transferred into fresh growth medium. Transfected cells were incubated with 5 μM CM-$H_2$DCFDA for 30 min at 37 °C, and then batches of cells were treated with various concentrations of AA (Sigma–Aldrich) for 30 min at 37 °C. After washing with phosphate-buffered saline (PBS), the DCF fluorescence intensity of DsRed2-nuc positive cells was immediately analyzed by flow cytometry (FACScan; Becton, Dickinson & Company Inc., Franklin Lakes, NJ, USA) according to the manufacturer's instructions. For each experiment, 10000 DsRed2-nuc positive events were collected and analyzed.

## 3. Results

### 3.1. Both AOX and E217A are expressed as non-covalently associated dimers in the mitochondria of HeLa cells grown in glucose and galactose medium

To perform the functional analysis of *Symplocarpus* AOX in human cells, we constructed an AOX plasmid, which encoded the mature form of *Symplocarpus* AOX fused to a human cytochrome *c* oxidase (COX) VIII mitochondrial targeting signal (MTS) [14] at its N terminus (Fig. 1A), and an E217A plasmid harboring a malfunctioning di-iron center generated by site-directed substitution of Glu-217 with Ala (Fig. 1A) [15] (Supplementary data). After transfection, cell proteins were immunoblotted under non-reducing conditions using an AOX monoclonal antibody raised against *S. guttatum* AOX [12]. A total of 24 h after the transfection with the control, AOX, or E217A plasmid, cytosolic and mitochondrial fractions were isolated from cells grown either in glucose or galactose medium. Fig. 1B shows that AOX proteins co-fractionated with the mitochondrial marker protein complex III subunit core 2. Furthermore, both of the mitochondrially targeted AOX proteins were predominantly expressed as a mature form, with a predicted molecular mass of 32 kDa, and there were fewer signals for the oxidized dimer in both mitochondrial fractions. The AOX protein with the extra MTS sequence was weakly detected in E217A-transfected mitochondria (Fig. 1B). In both cell growth mediums, E217A showed slightly more accumulation in the mitochondrial fraction than did AOX (Fig. 1B).

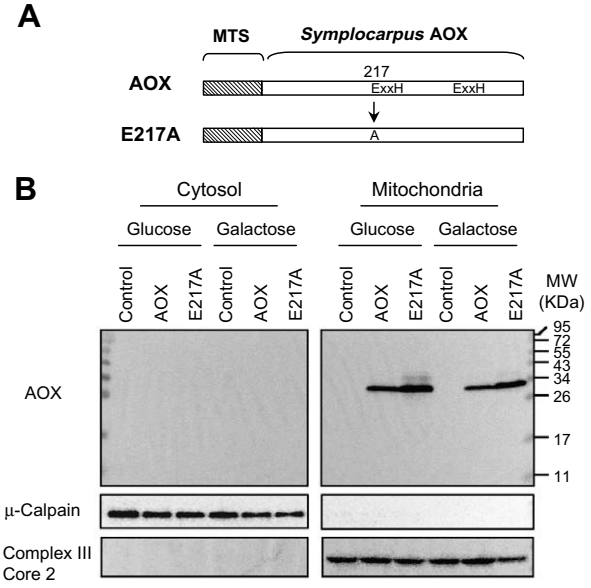


**Fig. 1.** Expression of AOX and E217A in HeLa cells. (A) Schematic representation of AOX and E217A constructs. Two highly conserved iron-binding motifs (EXXH) found in AOX species are shown. In E217A, Glu-217 of *Symplocarpus* AOX was replaced by Ala. MTS; mitochondrial targeting signal of human COX VIII. (B) Expression and subcellular localization of AOX and E217A in HeLa cells grown in glucose or galactose medium. Cytosolic and mitochondrial fractions (20 μg) from transfected cells were separated on 12.5% SDS–PAGE under non-reducing conditions and immunoblotted with the AOA monoclonal antibody against *S. guttatum* AOX [12], anti-μ-calpain (cytosolic marker), or anti-complex III core 2 subunit (mitochondrial marker). The positions of the molecular mass standards are indicated on the right of the panel. Data are representative results from two independent experiments.

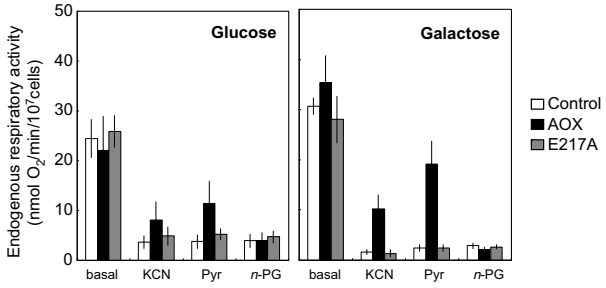


**Fig. 2.** HeLa cell endogenous oxygen consumption. Glucose- or galactose-grown cells were transfected with the empty vector (control), AOX, or E217A, and cellular oxygen consumptions were measured with buffers deprived of pyruvate at 37 °C. The data represent the mean ± S.E.M. of three independent experiments (nmol $O_2$/min per $10^7$ cells). KCN, 1 mM potassium cyanide; Pyr, 1 mM potassium pyruvate; *n*-PG, 0.1 mM *n*-propyl gallate.

### 3.2. AOX, but not E217A, confers cyanide-resistant respiration to HeLa cells

To determine whether AOX confers cyanide-resistant respiration to human cells, we measured rates of endogenous cellular oxygen consumption at 37 °C in a buffer lacking pyruvate. As shown in Fig. S1A (for glucose-grown) and S1B (for galactose-grown), 24 h after transfection with AOX (trace b), the cells exhibited substantial resistance to 1 mM cyanide and were virtually completely inhibited by the addition of 0.1 mM *n*-propyl gallate (*n*-PG), a specific inhibitor of AOX [16]. On the other hand, the oxygen consumption of control (trace a) and E217A-transfected (trace c) HeLa cells was not significantly affected by the addition of *n*-PG in the presence of cyanide. The percentages of basal endogenous oxygen consumption rates were 34.3 ± 4.4% and 28.6 ± 4.4% in glucose- and galactose-grown cells, respectively (Fig. 2, black bar). Addition of 1 mM pyruvate increased cyanide-resistant respiration more efficiently in AOX-expressing cells grown in galactose medium than in glucose medium (fold change of cyanide-resistant respiration by the addition of pyruvate was 1.5 ± 0.2-fold in glucose and 1.9 ± 0.1-fold in galactose, Fig. 2, black bar).

To further assess the effects of 1 mM pyruvate on cyanide-insensitive respiration in AOX-expressing mitochondria isolated from glucose-grown cells, we measured oxygen consumption at 37 °C using mitochondria from cells transfected with the control plasmid, AOX, or E217A. As shown in Fig. S2, in cells transfected with control (trace a) or E217A (trace c), addition of 0.5 mM KCN caused a dramatic decrease in respiration. However, progressive addition of 0.5 mM KCN to AOX-expressing mitochondria (trace b) resulted in significant cyanide-resistant and *n*-PG-sensitive respiration (Fig. S2). Moreover, addition of pyruvate (1 mM), but not α-ketoglutarate (1 mM) caused a 2.3 ± 0.2-fold ($n = 3$) increase in cyanide-insensitive respiration (Fig. S2, traces d and e). It should also be noted that there was no significant effect of heterologous expression of AOX on the basal and state 2 respirations (Table S1).

### 3.3. Intracellular pyruvate content in glucose-grown HeLa cells is higher than that of galactose-grown cells

Because pyruvate was less effective at stimulating AOX activity in glucose-grown cells, we next determined whether growth conditions (glucose or galactose medium) altered the levels of intracellular organic acids, in particular pyruvate. Our quantitative gas chromatography/mass spectrometry (GC/MS) measurements for eight different organic acids showed that intracellular pyruvate content of the glucose-grown cells was approximately four times higher than those detected in galactose-grown cells, whereas there was no significant difference in pyruvate contents between the control and AOX-expressing glucose-grown cells (Fig. S3). Moreover, AOX expression did not significantly affect the intracellular pools of other organic acids of cells grown in either growth medium (Fig. S3).

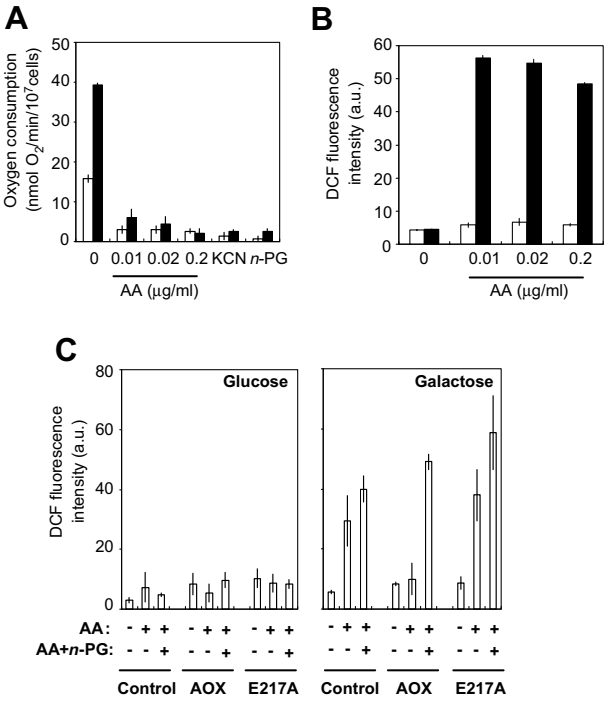


**Fig. 3.** Effects of antimycin A (AA) on oxygen consumption and ROS production in glucose- or galactose-grown HeLa cells. (A) The oxygen consumption of whole cells grown in either glucose or galactose was measured following treatment with various concentrations of AA at 37 °C. After treatment with AA, KCN (1 mM potassium cyanide) and *n*-PG (0.1 mM *n*-propyl gallate) were sequentially added to the media and oxygen consumption was measured. Open and filled bars denote glucose- and galactose-grown cells, respectively. (B) AA-induced ROS production in cells grown in media containing glucose or galactose. Cells were incubated with 5 μM CM-$H_2$DCFDA for 30 min at 37 °C, treated with various concentrations of AA for additional 30 min at 37 °C, and analyzed by flow cytometry. Open and filled bars denote glucose- and galactose-grown cells, respectively. (C) Effects of AA on AOX-expressing glucose- or galactose-grown cells. After 24 h of transfection with vector alone (control), AOX, or E217A, the cells were treated with 5 μM CM-$H_2$DCFDA for 30 min at 37 °C. Cells were further incubated with AA (0.01 μg/ml) for 30 min at 37 °C and analyzed by flow cytometry. Data from a representative experiment are shown and are the mean ± S.E.M. of triplicate determinations from three independent experiments.

### 3.4. AOX, but not E217A, diminishes AA-induced ROS production in galactose-grown HeLa cells

To determine the effects of AA on oxygen consumption and ROS production in glucose- and galactose-grown cells, cellular ROS generation was measured using the DCF fluorescence intensity and flow cytometry. As shown in Fig. 3A, oxygen consumption was severely inhibited in the presence of lower concentration of AA in both glucose- and galactose-grown cells (0.01 μg/ml). Addition of KCN and *n*-PG had no detectable effect on oxygen consumption, since these cells were not transfected with an AOX-expressing plasmid. ROS production, monitored by DCF fluorescence intensity, was significantly increased in galactose-grown cells incubated with AA (0.01, 0.02, and 0.2 μg/ml) (Fig. 3B).

We next determined the effect of AA-induced mitochondrial ROS production on AOX-expressing cells grown either in glucose or galactose media. If the AOX is sufficiently functional, the presence of AOX can prevent ROS generation by bypassing the AA-induced blockade of electron flow. No significant increase in DCF

fluorescence was observed in glucose-grown cells (Fig. 3C, left panel). However, in galactose-grown cells, cellular ROS production was increased in both control and E217A cells in the presence of AA (0.01 μg/ml) (Fig. 3C, right panel). On the contrary, AOX-expressing cells treated with AA (0.01 μg/ml) showed significant inhibition of ROS formation and DCF fluorescence levels were similar to those of untreated cells (control, AOX, or E217A) (Fig. 3C, right panel). ROS production in AOX-expressing cells was again observed following cell treatment with 0.1 mM *n*-PG (Fig. 3C, right panel).

## 4. Discussion

It was recently reported that ascidian AOX from *C. intestinalis* could be expressed in human cells and that it conferred mitochondrial cyanide resistance [10]. However, the authors concluded that the expression of the plant AOX in mammalian cells was unsuccessful due to uncontrolled lethality in the AOX-expressing cells [10]. Thus, although the significance of the AOX protein in mitochondrial metabolism has been extensively studied in higher plants, no functional analyses of plant AOXs in mammalian cells have been reported.

Here we demonstrate that plant *Symplocarpus* AOX can be successfully expressed in human cells, and that it confers a cyanide-resistant respiration pathway to human mitochondria. In addition, we show that *Symplocarpus* AOX clearly mitigates AA-induced ROS generation in mammalian cells. Interestingly, this AOX protective effect against AA-induced ROS formation was observed only in cells grown in galactose medium with more active respiration.

In our present study, cellular cyanide-resistant respiration was more effectively activated by the addition of 1 mM pyruvate in galactose-grown cells than in glucose-grown cells (Fig. 2). GC/MS analyses showed that the intracellular pyruvate pool in galactose-grown cells was apparently smaller than in glucose-grown cells (Fig. S3), suggesting that cyanide-resistant respiration may be more effectively activated by exogenous pyruvate in galactose-grown cells. It was recently reported that a switch from the M1 to the M2 isoform of pyruvate kinase in tumor cells resulted in an increase of pyruvate and lactate levels with reduced oxygen consumption of the cells [17]. Therefore, it is probable that pyruvate kinase M2, which is reported to be mainly expressed in glucose-grown HeLa cells [17], contributes to the increased pyruvate levels observed in these cells.

It has been suggested that ascidian AOX is also protective against AA-induced ROS formation in glucose-grown human embryonic kidney (HEK) 293 T cells based on an apparent decrease in SOD activity after incubation with 60 μM AA for 16 h [10]. However, these observations are complicated by the possibility of up-regulation of other ROS detoxification enzymes such as catalase and thioredoxin reductase [18]. Recent studies have also shown that higher concentrations of AA can bind not only cytochrome complex III at the Qi site, but also to the antioxidant protein Bcl-2 via its interaction with BH3 domains [19,20]. Thus, the current study using lower AA concentrations (0.01 μg/ml or 18.2 nM) and shorter periods of exposure (30 min) is the first report demonstrating that AOX plays a role in the prevention of ROS production in mammalian cells by direct measurement of AA-inducible ROS formation within the cells.

An intriguing question is why less ROS production was observed in AA-treated cells grown in glucose medium (Fig. 3C). The Warburg effect [21], which is known to repress mitochondrial respiration in glucose-grown cells (Fig. 3A), may be involved in this phenomenon. In accordance with this possibility, Wiesner and colleagues have shown that the combination of the mitochondrial uncoupler carbonyl cyanide m-chlorophenyl hydrazone (CCCP) with AA caused a significant elevation of ROS in glucose-grown HeLa cells, while AA alone had much smaller effects [22]. Another possible explanation is the involvement of glucose metabolism, which could potentially affect antioxidant activity within the cells. For example, glucose can induce SOD activity [23], produce excess pyruvate as an antioxidant (Fig. S3) [24], and produce intermediates of the pentose phosphate pathway, in which an antioxidant of the glutathione redox cycle, NADPH, is generated [25].

Rustin and colleagues noted that ascidian AOX was inactive under normal culture conditions [10], and they suggested that if AOX remains in a constitutively active form (as a non-covalently associated dimer), it would be detrimental to cell growth because of the associated decrease in ATP. Our results also show that despite the expression of AOX as a non-covalently associated dimer, the rate of basal oxygen consumption of AOX-expressing cells was not significantly enhanced either in glucose- or galactose-grown cells (Fig. 2). These results suggest that *Symplocarpus* AOX may have been less active under our culture conditions and that it was activated only under specific conditions when complex III was inhibited by AA.

Mitochondrial diseases have been generally attributed to dysfunctions in the cytochrome pathway, resulting in damage to organs and tissues with relatively high ATP demands, including the brain, heart, and skeletal muscle [26]. In particular, complex I deficiency is the most common cause of respiratory chain dysfunction [27]. Impairment of the ETC activity has also been observed not only in mitochondrial diseases, but also in Alzheimer's [28] and Parkinson's disease [29]. Moreover, complex III physically and functionally interacts with complexes I and IV [30], and its activity seems to have an influence on the other complexes [31]. Thus, our results further suggest that disorders and/or diseases associated with dysfunctional cytochrome segments, especially complexes III [32] and IV [26], could potentially be alleviated by introduction of *Symplocarpus* AOX as gene therapy within human mitochondria.

In summary, data presented in the present study clearly show that *Symplocarpus* AOX, which plays a pivotal role in skunk cabbage heat production, confers cyanide resistance to human cells and prevents ROS production induced by AA exposure. Functional expression of *Symplocarpus* AOX seems to be a valuable tool for understanding not only the plant enzyme but also the cytochrome pathway mutants associated with the human cell pathogenesis involved in tumorigenesis, apoptosis, and mitochondrial diseases.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.11.040.

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