A role for active oxygen species as second messengers in the induction of alternative oxidase gene expression in *Petunia hybrida* cells

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Abstract Incubation of *Petunia hybrida* cells with H_2O_2 leads to an increase in alternative oxidase activity measured after 24 h. This increased activity is accompanied by an increase in alternative oxidase protein. A model is presented for the regulation of alternative oxidase protein synthesis in which active oxygen species and especially H_2O_2 play a crucial role as second messengers in the signal transducing pathway from the mitochondria to the nucleus. It is proposed that also the induction of the alternative oxidase by salicylic acid is mediated via H_2O_2.

Key words: Alternative oxidase; Active oxygen species; Hydrogen peroxide; Plant mitochondria; Salicylic acid

1. Introduction

Besides the cytochrome pathway, plant mitochondria also contain a so-called alternative oxidase, which is identified as a quinol oxidase [1]. The alternative oxidase appears to be encoded by the nuclear gene *aox1* [2,3]. Little is known about the regulation of this gene expression. The developmental stage of the plant certainly plays a role: e.g. during flowering in *Araceae* the alternative oxidase is induced to a high extent. Also stress is an inducing factor in the expression. Chilling, wounding, drought and salt stress are examples of situations in which alternative oxidase is induced. A common factor in these situations is an inhibition of the cytochrome pathway [4]. Likewise, in tobacco and *Petunia hybrida* cell suspensions, expression of the alternative oxidase protein increases when the cytochrome pathway is inhibited with antimycin A [5–7]. The question is how the signal of a constricted cytochrome pathway is transduced to the nucleus. In this respect, active oxygen species and especially H_2O_2 have drawn our attention, because it was suggested that in fungi superoxide anions may be involved in the induction of the alternative oxidase [8]. It has been reported that upon addition of inhibitors of the respiratory pathways such as antimycin A, which blocks the cytochrome pathway, high rates of superoxide anions and H_2O_2 production in mitochondria can be observed [9,10]. Therefore the effect of H_2O_2 on the alternative respiration in *Petunia hybrida* cells was investigated.

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Abbreviations: BHAM, benzohydroxamate.

2. Materials and methods

2.1. Plant material

Petunia hybrida Vilm (cv Rosy Morn) cell suspensions were grown in batch culture as described by Van Emmerik et al. [11].

2.2. Determination of dry weight

Dry weight increase of the cell cultures was determined by separating the cells from the medium with a Büchner funnel. The cells were rinsed twice with distilled water and dried for at least 18 h at 60°C.

2.3. Isolation of mitochondria

Petunia hybrida mitochondria were isolated by homogenizing portions of 15 g cells (fresh weight) in 15 ml medium containing 0.4 M mannitol, 1 mM EDTA, 2 mM L-cystein, 0.2% BSA, 0.7% PVP-25 and 10 mM potassium phosphate buffer, pH 7.4, with a mortar and pestle at 4°C. (All further steps were performed at 4°C.) The homogenate was pressed through a double layer of cheese cloth and centrifuged at $1000 \times g$ for 5 min. The supernatant was centrifuged at $10,000 \times g$ for 10 min. The pellet was washed once with a washing medium containing 0.4 M mannitol, 0.2% BSA, and 10 mM potassium phosphate buffer, pH 7.2. The pellet was resuspended in 2 ml washing medium and loaded on a Percoll gradient (21% Percoll in washing medium). After centrifugation for 30 min at $20,000 \times g$ the mitochondrial band was collected, diluted 25 times with washing medium and centrifuged for 10 min at $10,000 \times g$. The pellet was suspended in 1–2 ml washing medium.

2.4. Measurements of respiration

Respiration of cells and mitochondria was measured at 25°C using a YSI oxygen monitor model 53 and a 5 ml (cells) or 1 ml (mitochondria) reaction vessel. Mitochondrial respiration was measured in a reaction medium containing 0.4 M mannitol, 10 mM potassium phosphate buffer, 0.2% BSA, pH 7.1 with a mixture of succinate (20 mM) and NADH (2 mM) as substrates. For state 3 measurements 0.1 mM ADP was supplied. Respiration of cells was measured by transferring 5 ml of the cell suspension to the reaction vessel. The alternative pathway was inhibited with benzohydroxamate (BHAM, 2 and 13 mM for mitochondria and cells, respectively) and the cytochrome pathway was inhibited with KCN (0.1 mM and 0.35 mM for mitochondria and cells, respectively).

Mitochondrial protein was determined with the Bradford method [12].

2.5. SDS-PAGE and immunoblotting

Up to 100 μg of mitochondrial protein was solubilized in sample buffer (312 mM Tris [pH 6.8], 10% [w/v] SDS, 10% [v/v] glycerol, 0.002% [w/v] bromphenol blue and 100 mM DTT) and boiled for 1–2 min. The mitochondrial samples were subjected to SDS-polyacylamide electrophoresis (10% gel), followed by Western blotting. Antibodies developed against the alternative oxidase protein of *Sauromatum guttatum* (generously supplied by Dr. T. Elthon) were used at dilutions of 1:1000. Visualization was with the ECL chemiluminescent reagent system (Amersham).

3. Results and discussion

In *Petunia hybrida* cell suspension cultures, an increase in total respiration and CN-resistant respiration occurs after subculturing the cells in fresh medium. This respiratory peak lasts for about two days after which the levels decline to the situation

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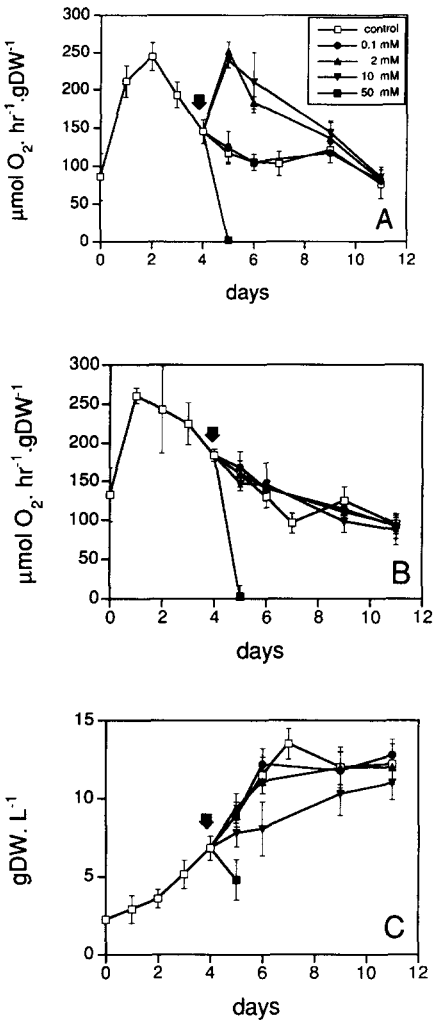

Fig. 1. The course of CN-resistant respiration (A), uninhibited, total respiration (B) and dry weight increase (C) in *Petunia hybrida* cells grown in the absence (□) or presence of 0.1 mM (●), 2 mM (▲), 10 mM (▼) or 50 mM (■) H_2O_2. H_2O_2 was added on day 4 (indicated by the arrows). Data are mean values of three different batch cycles. Bars represent SD.

before inoculation [11]. H_2O_2 (added at day 4, when the initial respiratory peak had disappeared) influenced the CN-resistant respiration, with the effect dependent on the concentration given. Addition of 2 mM and 10 mM H_2O_2 both resulted in an increased CN-resistant respiration. This increased activity remained present in the cells for 4–5 days, after that period CN-resistant respiration was no longer different from the control cells (Fig. 1A). With 0.1 mM H_2O_2 no effect was visible, but since it was observed that the H_2O_2 rapidly disappeared from the culture medium after addition (possibly via catalase present in the cells and medium) it might be that such low concentrations do not persist long enough to have an effect. After addition of 50 mM H_2O_2 browning of the cells was observed and CN-resistant respiration was strongly inhibited (Fig. 1A). In these cells total uninhibited respiration was also severely effected, indicating a lethal effect of such high concentrations. Addition of 10 mM, 2 mM or 0.1 mM H_2O_2 did not effect uninhibited respiration (Fig. 1B). Growth of the cells stopped after addition of 50 mM H_2O_2 to the cells, and was somewhat slower with 10 mM H_2O_2 present in the medium than in the control cells (Fig. 1C). It is not clear yet whether this is caused by a decrease in growth rate of all cells or by the death of some of the cells. Addition of 0.1 mM and 2 mM H_2O_2 did not influence the growth rate.

Also in mitochondria (Fig. 2A,B), isolated from H_2O_2 treated cells, CN-resistant respiration was increased. Total respiration, measured 24 h after addition of 2 mM H_2O_2 was somewhat higher, especially in state 4, than in control mitochondria and RC ratios were lower, but in the presence of BHAM respiration and RC ratios were similar to the control mitochondria (Fig. 2C), indicating that in mitochondria from H_2O_2 treated cells the alternative pathway was engaged in uninhibited oxygen uptake, but that the cytochrome pathway was not influenced by the treatment. Accordingly, cytochrome oxidase activity was not increased after treatment with H_2O_2 (data not shown).

An increase of the O_2 uptake via the alternative pathway can be caused either by extra protein synthesis or by activation (via organic acids [13–16] and/or thiol modifications in the enzyme [17,18]). However, after 1 h in the presence of 10 mM H_2O_2, CN-resistant respiration was not different from the control cells (data not shown), indicating that extra protein synthesis and not activation of already existing protein is involved in the increase in activity. Western blots of H_2O_2 treated and control cells (Fig. 3) indeed showed that extra alternative oxidase protein is present after 24 h (but not after 1 h) in the presence of H_2O_2.

To our knowledge this is the first time that it is described that in higher plants, as in fungi [8], H_2O_2 can induce alternative oxidase gene expression. Active oxygen species like H_2O_2 are formed under conditions when the cytochrome pathway is impaired [4,8]. These products are a great danger to the cell, because they can initiate lipid peroxidation and consequently membrane damage occurs. Purvis and Shewfelt [4] recently suggested that engagement of the alternative pathway helps to maintain reduction of respiratory components low, thereby preventing excessive production of free radicals. Indeed, determination of in vivo Q reduction levels [19] showed that these values are maintained at a rather stable level. It is tempting to suggest that we finally have found a function for this until now

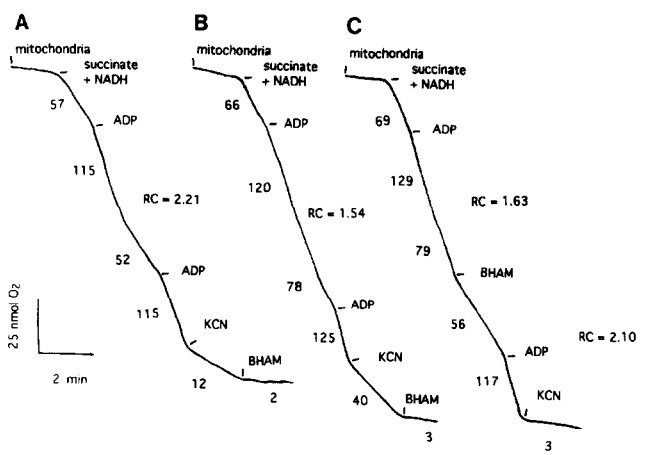

Fig. 2. Oxygen uptake of mitochondria isolated from *Petunia hybrida* cells incubated for 24 h in the absence (A) or presence (B,C) of 2 mM H_2O_2. As indicated, 20 mM succinate, 2 mM NADH, 0.15 mM ADP, 0.1 mM KCN, and 2 mM BHAM were added. Values refer to µmol $O_2 \cdot h^{-1} \cdot$ mg protein^{-1}.

rather mystical electron transport pathway to oxygen. In this view, both the activation of the alternative pathway and induction of alternative oxidase gene expression contribute to the protection of the plant against active oxygen species. When substrate supply to the mitochondria is too high for the (impaired) cytochrome pathway, accumulating organic acids lower the K_m of the oxidase for ubiquinol [13–16,18], and the increased activity of the pathway will prevent an increase of reduction levels of the respiratory components, which would lead to the formation of harmful active oxygen species. Whenever the (activated) capacity of the alternative oxidase is not sufficient and active oxygen species like H_2O_2 do accumulate, these products themselves induce the expression of extra alternative oxidase protein. A messenger function for active oxygen species has been proposed before in the defence mechanism of plants [20]. Recently Chen [21] showed that H_2O_2 acts as a second messenger in the expression of the so-called PR proteins that are involved in the systemic acquired resistance response of plants. Salicylic acid plays an important role in this induction. Salicylic acid binds to a protein, which appears to be a catalase. Binding of salicylic acid inhibits catalase activity, leading to increased levels of H_2O_2 in the cells, which in turn stimulate gene expression via activation of a transcription factor, as has been described in animal systems [21,22]. Also in other plant species, salicylic acid inhibits catalase activity [23]. It seems too much of a coincidence that on the one hand H_2O_2 can induce alternative oxidase gene expression in higher plants, while on the other hand salicylic acid is involved in the induction of the alternative oxidase in flowering Araceae spadices [24], in tobacco cell suspensions [25,26] and potato [27]. We therefore propose that also in the salicylic acid mediated induction of the alternative oxidase active oxygen species act as second messengers, produced by binding of salicylic acid to a catalase. The observation of Rhoads and McIntosh [28], that

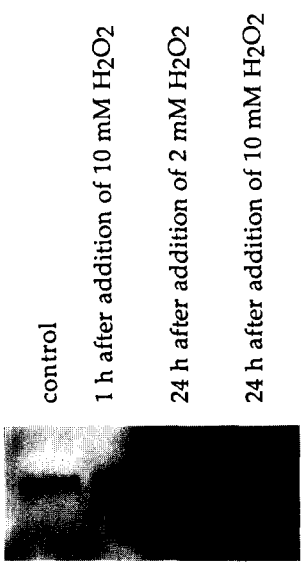

Fig. 3. The levels of the 35 kDa alternative oxidase protein in mitochondria from *Petunia hybrida* cells. From left to right: control; 1 h after addition of 10 mM H_2O_2 to the cells; 24 h after addition of 2 mM H_2O_2 to the cells; 24 h after addition of 10 mM H_2O_2 to the cells. Mitochondrial protein (100 μg in each lane) was separated by SDS-PAGE, transferred to nitrocellulose and probed with monoclonal antibodies against the alternative oxidase.

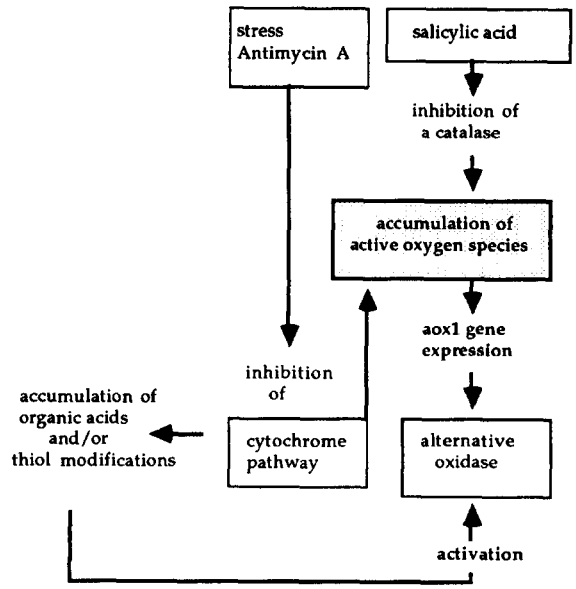

Fig. 4. Putative scheme for the activation and induction of the alternative oxidase protein, and the central role of active oxygen species.

the salicylic acid-inducible alternative oxidase gene *aox1* and genes encoding pathogenesis-related proteins share regions of sequence similarity in their promoters fit very well into this model. Fig. 4 shows a putative scheme of the mechanisms that may be involved in the induction and activation of the alternative oxidase.

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